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This is a request for International Application **PCT/EP99/07127** to enter U.S. national stage under 35 U.S.C. § 371. Commencement of **national** stage processing of this application is expressly requested under § 371(f).

International filing date: **27 September 1999**

Earliest priority date claimed: **26 March 1999**

Title: **DETECTION OF NUCLEIC ACID AMPLIFIED PRODUCTS**

Inventor(s)(full name of each inventor): **KRUPP, Guido**

Items required upon filing:

X Return receipt postcard

X Copy of the PCT application

____ pages of the PCT application enclosed.

A copy of the PCT application does not need to be filed if a pink Form PCT/IB/308 has been received in the PCT application and it gives notice that the International Bureau has communicated a copy of the application the U.S. designated office.

X Form PCT/IB/308 was mailed on **05 October 2000**

X English translation of claims accompanying the International Preliminary Examination Report (IPER) mailed **May 10, 2001**.

X A check in the amount of **$ 1182.00** is enclosed to cover the Basic National fee and any Excess Claim fees, calculated as follows (payment of the fee cannot be deferred until after filing)

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	Number Filed		Number Extra		Rate: Small Entity	Rate: Other Entity		Fee: Small Entity	Fee: Other Entity
Total Claims	29-20	=	9	x	$9	$18	=	$	$162
Independent Claims	5-3	=	2	x	$40	$80	=	$	$160
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Items that are optional or may be deferred:

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Sequence listing in computer readable form
Statement Under § 1.821(f)

____ Translation of the non-English application
____ Amendments to the PCT application under Article 19 (English translation)
____ Preliminary Amendment
____ Also enclosed: ______________________________

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Detection of nucleic acid amplified products

The present patent application relates in particular to processes for the amplification and quantitative real-time detection of nucleic acids as well as kits for carrying out the processes.

Various nucleic acid amplification techniques (NAT), such as for example polymerase chain reaction (PCR) or nucleic acid sequence-based amplification (NASBA®), have been developed to date for the multiplication of deoxyribonucleic acids (DNA) or ribonucleic acids (RNA). Assays based on these amplification techniques are used for example for the highly-sensitive detection and/or the quantification of pathogens in the medical-diagnostic field.

DNA amplification techniques such as PCR lead to the production of large quantities of amplified target DNA (or via an initial reverse transcriptase step to amplified RNA). Usually, the amplification products are detected after a defined period by means of post-amplification methods - in general through hybridization (end-point analysis).

According to a new approach - "TaqMan®" - for quantitative PCR, fluorescence resonance transfer (FRET; cf. Heid et al., Genome Res. 6 (1996) 986-994) with double-fluorescence-marked DNA

probes is proposed for real-time detection of DNA amplification. A disadvantage of this method is that the probe adheres to the target until it is removed by the 5' exonuclease activity of the Taq DNA polymerase. Due to the temperature profile of the PCR, stringency can be controlled only with great difficulty, and the solution of this problem through appropriate probe design is conceivable only with major outlay. A further disadvantage of TaqMan® is the production of an equimolar signal, i.e. that only one probe molecule is split per amplified DNA target molecule per amplification cycle, which results in a comparatively weak signal.

NASBA® - in contrast to thermocyclical PCR - is a homogenous, isothermal *in vitro* amplification (cf. e.g. T. Kievits et al, J. Vir. Meth. 35 (1991) 273-286), EP 0 329 822 as well as R. Sooknanan et al. in "Molecular Methods for Virus Detection", D.L. Wiedbrauk and D.H. Farkas (Ed.), Academic Press 1995, chapter 12, 261-285). Compared with other amplification processes, NASBA® and other isothermal reactions have the advantage that they can be carried out without particular technical outlay, as the amplification takes place at a single temperature value and these reaction conditions are retained during the whole process. The duration of each amplification step is thus not shortened either. In conjunction with the amplification efficiency, high e.g. compared with PCR, high amplified fragment concentrations are thus achieved in a short time by means of NASBA® and other isothermal amplification techniques. A further advantage of NASBA® compared with PCR results from the selective detection potential of RNA. This is important in particular in connection with the amplification or quantification of cellular mRNA, with which possible cellular DNA contaminations can be avoided.

A disadvantage of NASBA® and other isothermal amplification strategies is however that a real-time detection by means of fluorescence, such as with the PCR-based TaqMan® (Perkin Elmer) or Light-Cycler (Roche Diagnostics) is not possible.

There are problems with the end-point analysis proposed in this connection for quantification as, in the case of detection of different target-RNA concentrations, some samples may already have reached saturation level (plateau phase), whilst other samples are still in the phase of increasing amplified fragment concentrations (cf. also Heid et al, op cit). Furthermore, this end-point analysis is more expensive and time-consuming due to additional work steps following the RNA amplification. Due to the need to open the reaction vessels for the quantification steps, there is also the risk of a cross-contamination of highly amplified RNA and DNA targets.

Leone et al. (Nucleic Acids Research 26 (1998) 2150-2155) proposed an approach to the real-time detection of NASBA®-amplified RNA in which a double-fluorescence-marked DNA probe is used. In contrast to the PCR process (cf. Heid et al., op cit), the probe adheres to the target and is not removed during the amplification reaction. This leads to potential complications, as the DNA probes can interfere during the early amplification stages with the binding to the first antisense-RNA amplified fragments, which can lead to decomposition by Rnase H and thus to the elimination of RNA substrates and consequently to an erroneous concentration determination. Moreover the accuracy of the quantitative target determination depends decisively on the quantity of probe added.

However, the system proposed by Leone et al. allows only a very poor quantification irrespective of whether the preferred evaluation is carried out based on the threshold value (cf. Leone et al., Figure 7; curves for 100 fg and 1 pg overlap at the beginning) or after reaching the plateau (cf. Leone et al., Figure 7; curves for 1 pg and 10 pg overlap at the end).

Furthermore, only a very low stringency is possible, as the probe adheres to the target and the isothermal reaction takes place at a relatively low temperature (41°C),

which results in a high risk of falsely positive results. Obviously, a maximum signal could be obtained even at low temperatures, depending on the probe (cf. Leone et al., Figure 7), but due to the test procedure chosen, this would result in an additional risk of falsely positive results. As was ascertained within the framework of further studies using the protocol proposed by Leone et al., the optimum temperature for the hybridization of the fluorescence marker varies depending on the length or the sequence of the hybridizing target section.

The object of the present invention is therefore to provide a process for the real-time detection of nucleic acids, in particular of RNA, which avoids the disadvantages of the methods known in the state of the art, in particular of the process of Leone et al, and is suitable for routine applications.

The object is achieved according to the invention through processes according to claims 1 to 5.

The present invention thus relates to a process for the amplification and quantitative real-time detection of nucleic acids in which

a) a primer is used to which a nucleic acid sequence, preferably with a length of 1 to 40 nucleotides, is attached, which codes for the sequence motif 5'-GAAA-3' (motif A) in the transcript,

b) the amplification being carried out in the presence of an excess, preferably in a concentration of 50 to 500 nM, of a nucleic acid probe, preferably with a length of 25 to 60 nucleotides (particularly preferably approx. 50 nucleotides) which contains the sequence motif 5'-CUGANGA-3' (motif B), a reporter molecule and a quencher molecule being attached to each probe molecule, and

c) the original concentration of the nucleic acid in the sample is determined by measuring the time-dependent change in fluorescence during amplification, the relative concentration "$C_{rel.}$" being determined according to the following formula:

$$C_{rel.}=t_{L}/t_{Ref.}$$

where

t_p corresponds to the time measured for the sample from the start of amplification to the reaching of the fluorescence threshold value and

$t_{Ref.}$ corresponds to time measured for a reference nucleic acid of known concentration from the start of amplification to the reaching of the fluorescence threshold value.

The process according to the invention which, due to the sequence motif A introduced via the primer or attached to the amplified nucleic acid fragments and the motif B used in the probe, makes the formation of a hammerhead ribozyme possible, involves the cleavage of the probe and thus the production of a fluorescence signal. The principle according to the invention is shown schematically in Fig. 1 (and Figs. 2 to 16). According to the invention, it is of course possible to exploit sequences which, instead of the hammerhead ribozyme, are suitable for the development of other, smaller ribozymes (e.g. the "hairpin-ribozyme" or the "hepatitis delta").

The process according to the invention is particularly suitable for the quantification of RNA, DNA or RNA/DNA chimeras (i.e. nucleic acids containing ribo- and deoxyribonucleotides) which are called "target nucleic acid", where a melting of double-stranded nucleic acids upstream from the process may be necessary to obtain single strands.

The amplification processes suitable within the framework of the present invention are preferably isothermal amplification processes such as NASBA®, transcription mediated amplification (TMA; cf. e.g. M. Hirose et al, J. Clin. Microbiol. 36 (1998) 3122-6) or self-sustained sequence replication (3SR; cf. E. Fahy et al. in PCR Methods and Applications, Cold Spring Harbor Laboratory Press 1991, 25-33) or cyclical amplification processes such as e.g. PCR.

Unless otherwise indicated herein, the nucleotides A, C and G can each be ribonucleotides (rNTP) or deoxyribonucleotides (dNTP). "N" can stand for any ribo- or dexoyribonucleotide. In the case of RNA/DNA chimeras (i.e. oligonucleotides which contain both ribo- and deoxyribonucleotides), the obligatory ribonucleotides are provided with the prefix "r" (e.g. rA, rC. rG) or U. The sequence motifs A and B of the probes can thus consist either exclusively of ribonucleotides (RNA probe) or RNA/DNA chimeras. In the case of motif A, it is necessary however that the ribonucleotide adenine (rA) be used at the 3' end in each case (i.e. 5'-GAA(rA)-3'). In the case of motif B (5'-CUGANGA-3'), it is necessary that guanine be present as ribonucleotide and adenine also be a ribonucleotide (rA) at the 3' end (i.e. 5'-CU(rG)AN(rG)(rA)-3'). U can optionally be replaced by T.

By "fluorescence threshold value" is meant within the framework of the present invention a fluorescence value which exceeds by a factor of 5-10 the background fluctuation measured under comparable conditions (i.e. reaction mixture without target or reference nucleic acid).

The time t_p corresponds to the time which elapses after the start of the amplification reaction until so many amplified fragments of the target nucleic acid have formed that the fluorescence threshold value (threshold value) is reached.

The time $t_{Ref.}$ corresponds to the time which elapses after the start of the amplification reaction until, starting from a reference nucleic acid of known concentration, so many amplified fragments have formed that the threshold value is reached. The reference nucleic acid should deviate only slightly in its nucleic acid sequence from the target nucleic acid sequence so that as accurate a quantification as possible is achieved.

To be able to determine the concentration of the target nucleic acid as accurately as possible, several $t_{Ref.}$ values for reference nucleic acids of different concentration are preferably measured so that the measured t_p value lies if possible between two $t_{Ref.}$ measurement points and thus a specified concentration can be allocated. Preferably, three t_{Ref} values are measured for one reference nucleic acid at three different concentrations and the measurement curve (standard curve) resulting from this established. The target nucleic acid of unknown concentration can then be determined after determination of the t_p value by comparison with the standard curve.

According to a particular version of the invention, the process is carried out by using the target nucleic acid in simultaneous presence of one or more, preferably of three reference nucleic acids of known concentration, and for detection, different sequence-specific, fluoresence-marked probes which produce a different fluorescence signal. The sequences of the reference nucleic acids in one amplification set differ only slightly from each other and should be variants of the target nucleic acid. In this way, the t_p and $t_{Ref.}$ values in one reaction set can be determined simultaneously and thus the concentration ($C_{Rel.}$) of the target nucleic acid determined without additional operating outlay (so-called "Multiplexing"; cf. also US 5,837,501).

Instead of the use of a primer containing the sequence motif A and a probe containing the sequence motif B, the reverse combination is also equally suitable, i.e. the

combination of a primer containing the motif B and a probe containing the motif A.

Practically all fluorescence dyes and in particular the dyes given in Tab. III (above all FAM, HEX, TET, ALEXA, Texas Red, Light Cycler Red, IRD 700, CY-7, IRD 41 or La Jolla Blue (TIB MOLBIOL)) come into consideration as reporters. Preferably, the reporter dyes are substances with a high fluorescence signal (i.e. high "light yield") and a low "photobleaching".

As quenchers, dyes can be used which absorb at wavelengths of > approx. 500 nm. Among the substances coming into consideration, TAMRA, LCR, CY-5 or DABCYL are preferred.

Within the framework of the present invention, reporter/quencher combinations are preferred which allow an excitation at approx. 490 nm and an emission at < approx. 650 nm (TaqMan® SDS 7700, Perkin Elmer) or < 700 (Light Cycler, Boehringer). The fluorescence can be measured with practically every fluorimeter customary in the trade.

For multiplexing, the combination of the universal quencher DABCYL with reporter dyes such as cumarin (emitted fluorescence at 475 nm), FAM (emitted fluorescence at 515 nm), BODIPY (emitted fluorescence at 525 nm), TAMRA (emitted fluorescence at 575 nm), Texas Red (emitted fluorescence at 615 nm), CY-5 (emitted fluorescence at 674 nm) etc. suggests itself (cf. e.g. S. Tyagi et al., Nature Biotech. 16 (1998) 49-53).

Should the nucleic acid to be amplified already contain the sequence motifs 5'-GAAA-3' or 5'-CUGANGA-3' ("ribozyme motifs"), the process for amplification and quantitative real-time detection can also be carried out according to the invention; because of the ribozyme motif already contained in the target nucleic acid, unmarked primers are used, i.e. primers to which motif A or motif B are not attached. The

detection finally takes place by carrying out the nucleic acid amplification - preferably NASBA®, TMA, 3SR or PCR - in the presence of an excess of a probe which contains the motif "complementary" to the ribozyme motif contained in the target nucleic acid in each case. By "complementary motif" is meant within the framework of the present invention a motif which - depending on the ribozyme motif contained in the target RNA (5'-GAAA-3' or 5'-CUGANGA-3') - is necessary for the development of a hammerhead ribozyme structure (hammerhead ribozyme).

The present invention thus relates to a process for the amplification and quantitative real-time detection of a nucleic acid containing the sequence motif 5'-GAAA-3' (motif A) or the sequence motif 5'-CUGANGA-3' (motif B), in which

a) the sequences of the primers used are chosen such that the sequence range of the nucleic acid which codes for the motif A in the transcript is amplified,

b) the amplification being carried out in the presence of an excess, preferably in a concentration of 50 to 500 nM, of a nucleic acid probe, preferably with a length of 25 to 60 nucleotides (particularly preferably approx. 50 nucleotides) which contains the sequence motif 5'-CUGANGA-3' (motif B) or the sequence motif 5'-GAAA-3' (motif A), a reporter molecule and a quencher molecule being attached to each probe molecule, and

c) the original concentration of the nucleic acid in the sample is determined by measuring the time-dependent change in fluorescence during amplification, the relative concentration "$C_{rel.}$" being determined according to the following formula:

$$C_{rel.}=t_r/t_{Ref.}$$

where

t_p corresponds to the time measured for the sample from the start of the amplification to the reaching of the fluorescence threshold value and

$t_{Ref.}$ corresponds to the time measured for a reference nucleic acid of known concentration from the start of the amplification to the reaching of the fluorescence threshold value.

With the process according to the invention, a quantitative real-time detection of nucleic acids (i.e. RNA, DNA or RNA-DNA chimeras) within the framework of an isothermal nucleic acid amplification, e.g. by means of NASBA®, TMA or 3SR, is thus possible for the first time. In the case of NASBA®, in particular the problems affecting the system of Leone et al. (op cit) are avoided. Furthermore, there is no possible competition between detection and amplification as the probe - an RNA substrate probe - does not adhere to the target but is split off and released, whereby a detectable signal is generated. Furthermore, it is advantageous that Rnase H cannot decompose the target RNA in the hybrid comprising RNA substrate probe and RNA target. Furthermore, the quantity of RNA substrate probe is not critical, and it can be used in a very large excess, such as e.g. 500 nM vis-à-vis 2 nM ribozyme target or 0.066 nM ribozyme.

Compared with the PCR-based real-time processes such as TaqMan® or Light Cycler®, the process according to the invention also has advantages under isothermal and cyclical temperature conditions (PCR). Due to the possibility of cleaving several probes within the framework of one amplification step, a comparatively higher signal can be generated. This leads to a higher sensitivity of the reaction and to a shortened reaction time. In addition, the signal generation is in principle controllable due to the enzymatic cleavage. A further advantage of the described process lies in the high specificity

of the reaction, as only an exact hybridization of the probe with the target sequence leads to the cleavage process and thus to the formation of a significant signal. Furthermore, in particular compared with TaqMan®, no costly probe design is necessary, as the probe detaches itself from the target sequence after each cleavage process. A further advantage of the described process lies in the possibility of multiplexing.

The process according to the invention allows a very good and exact linear quantification due to the enzymatic cleavage of the probe. In the ribosystem according to the invention, the hybridization itself produces only a very weak signal, whilst every ribozyme present in the amplified nucleic acid splits a large number of nucleic acid substrate probes. This further amplification is very specific and requires the presence of a completely hybridizing sequence (cf. Singh et al., Antisense and Nucleic Acid Drug Dev. 6 (1996) 165-168). Without the risk of obtaining falsely positive results, temperature and other reaction conditions can be optimized in order to achieve a maximum fluorescence signal. For example, synthetic peptides (cf. Müller et al., J. Mol. Biol. 242 (1994) 422-429), CTAB (Nedbal et al., Biochemistry 36 (1997) 13552-7) or GAP-DH (Sioud et al., J. Mol. Biol. 257 (1996) 775-789) can be added which can increase efficiency, such as e.g. the hybridization speed and the specificity of target recognition.

Compared with the amplification processes used or proposed in the state of the art with target quantification, the stability of the RNA probe can be increased by the present invention and its costs simultaneously reduced. Thus it is e.g. possible to replace almost all ribonucleotides, more expensive in chemical synthesis, with 2'-deoxyribonucleotides which are cheaper and more resistant to decomposition (through longer-term storage, exposure to nucleases, metal ions such as magnesium, as well as heat etc.; cf. Bratty et al., Biochim. Biophys. Acta 1216 (1993) 345-359).

With regard to an improvement in the general ribozyme structure and efficiency of the process, the following modifications are possible, among others:

To increase the reaction speed, i.e. to produce more signals relative to the number of amplified nucleic acid molecules, the sequence UA should follow the cleavage site of the ribozyme (cf. Clouet-d'Orval et al, Biochemistry 36 (1997) 9087-9092). Moreover, the position X (cf. Figure 4B) should contain the modified base pyridine-4-on (cf. Burgin et al., Biochemistry 35 (1996) 14090-14097), which likewise leads to an increase in the reaction speed of the detection stage.

By replacing most ribonucleotides with deoxyribonucleotides, the costs for an RNA probe can be reduced by up to 10 times. Ribonucleotides are however essential at four positions, which are identified by "r" e.g. in Fig. 2B, 4B, 15 and 16 (cf. Byang et al, Biochemistry 31 (1992) 5005-5009). Further, in the tables present therein, to distinguish between deoxy- and ribonucleotides, upper case letters are used (for dNTPs) and lower case (for rNTPs).

Furthermore, it has been shown that chimeric DNA/RNA hammerhead ribozymes have an increased catalytic efficient and stability (N.R. Taylor et al, Nucleic Acids Research 20 (1992) 4559-4565). This principle can be exploited according to the invention in particular for amplification processes such as e.g. PCR which are carried out at higher temperatures or with cyclical temperature profiles.

Additives such as e.g. the protein GAP-DH (cf. Sioud et al., J. Mol. Biol. 257 (1996) 776-789), short synthetic peptides which are derived from the viral coat protein (cf. Müller et al., J. Mol. Biol. 242 (1994) 422-429) or the chemical substance CTAB (Netbal et al., Biochemistry 36 (1997) 13552-13557) are suitable for increasing the effectiveness of the process with regard to the discovery of targets "hidden" in large nucleic acid structures, i.e.

ribozyme motifs.

On the basis of the present invention, it is possible for the first time to detect several different targets simultaneously by using corresponding ribozyme probes with different reporter dyes. Sequence-specific probes are necessary which adhere selectively to the target nucleic acids to be detected in each case and produce fluorescence signals of different wavelength during ribozyme cleavage. For example, it is possible to combine the quencher DABCYL with reporter dyes such as e.g. cumarin (fluorescence emission at 475 nm), FAM (fluorescence emission at 515 nm), BODIPY (fluorescence emission at 525 nm), TAMRA (fluorescence emission at 575 nm), Texas Red (615 nm), CY-5 (674 nm) etc. (cf. Tyagi et al., Nature Biotech. 16 (1998) 49-53). It is thus possible with this so-called "multiplexing" to simultaneously amplify within one reaction set a target RNA and several reference samples of known concentration, the sequences of which differ slightly from one another in the primer-binding section in each case, a quantification being able to take place through sequence-specific probes which carry different reporter/quencher combinations, without separate amplifications and fluorescence measurements having to be carried out with the RNA reference samples.

The present invention furthermore relates to a kit for carrying out the above-named processes which comprises either

a) an amplification primer to which a nucleic acid sequence, preferably with a length of 1 to 40 nucleotides, is attached, which codes for the sequence motif 5'-GAAA-3' (or 5'-CUGANGA-3') in the transcript,
b) a further amplification primer,
c) enzymes and reagents for carrying out the amplification reaction,
d) a nucleic acid probe, preferably with a length of 25 to 60 nucleotides (particularly preferably approx. 50

nucleotides) which contains the sequence motif 5'-CUGANGA-3' (or 5'-GAAA-3'), a reporter molecule and a quencher molecule being attached to each probe molecule, as well as optionally

e) apparatus and auxiliaries necessary for carrying out the reaction,

or

a) two amplification primers,

b) enzymes for carrying out the amplification,

c) a nucleic acid probe, preferably with a length of 25 to 60 nucleotides (particularly preferably approx. 50 nucleotides), which contains the sequence motif 5'-CUGANGA-3' (or 5'-GAAA-3'), a reporter molecule and a quencher molecule being attached to each probe molecule, as well as optionally

d) apparatus and auxiliaries necessary for carrying out the reaction.

According to a partial aspect of the present invention, for the first time, a process for the detection of nucleic acids as well as kits for carrying out the process are made available.

In particular, the invention relates to a process for detecting nucleic acids which contain the sequence motif 5'-GAAA-3' (motif A) or the sequence motif 5'-CUGANGA-3' (motif B), in which a sample containing the nucleic acid is brought into contact with a probe, preferably with a length of 25 to 60 nucleotides (particularly preferably approx. 50 nucleotides) which contains the sequence motif 5'-CUGANGA-3' (motif B) or the sequence motif 5'-GAAA-3' (motif A), a reporter molecule and a quencher molecule being attached to each probe molecule, the probe having to contain a sequence suitable for the hybridization with the nucleic acid to be detected and the nucleic acid

being detected by obtaining a fluorescence signal corresponding to the choice of reporter and quencher molecules.

A kit according to the invention for carrying out this detection process comprises in addition to solvent and reagents necessary for carrying out the reaction, a probe, preferably with a length of 25 to 60 nucleotides (particularly preferably approx. 50 nucleotides) which contains the sequence motif 5'-CUGANGA-3' (motif B) or the sequence motif 5'-GAAA-3' (motif A), a reporter molecule and a quencher molecule (see above) being attached to each probe molecule, the probe having to contain a sequence suitable for the hybridization with the nucleic acid to be detected.

In the event that the target nucleic acids contain none of the sequence motifs A or B, the nucleic acid can be detected by introducing one of the motifs e.g. through nucleic acid amplification using a primer named above. A corresponding double-fluorescence-marked probe (see above) which contains a sequence motif suitable for ribozyme formation is necessary for detection.

With the process and kits according to the invention - with or without use of a nucleic acid amplification - a new method for pathogen detection is made available. As indicated in the following, for example the 16S rRNA of many pathogen species already naturally contains a 5-'GAAA-3' ribozyme motif which can be used to form the hammerhead ribozyme. If the nucleic acids of the pathogens contain none of the sequence motifs suitable for the development of ribozymes, the former can, as indicated above, be introduced or "added" within the framework of the amplification stages by using suitable primers.

Table 1: <u>GAAA in 16S rRNA</u>

Region in E.coli 16S rRNA	70-100	115-145
E.coli	---	taatgtctgg**GAAA**ctgcctgatg
Salmonella	---	taatgtctgg**GAAA**ctgcctgatg
Staphylococcus	---	---
C. perfringens	tttccttcgg**GAAA**cggattagcg	---
Vibrio	aagtcgagcg**GAAA**cgagttatct	taatgcctag**GAAA**ttgccctgat
B. cereus	---	---
C. botulinum	---	---
Campylobacter	---	---
Yersinia	---	taatgtctgg**GAAA**ctgcctgatg
Listeria	---	---

Region in E.coli 16S rRNA	145-175	180-210
E.coli	ataactactg**GAAA**cggtagctaa	---
Salmonella	ataactactg**GAAA**cggtggctaa	---
Staphylococcus	ataacttcgg**GAAA**ccggagctaa	gttcaaaagt**GAAA**gacggtcttg
C. perfringens	atagccttcc**GAAA**ggaagattaa	tcataatgtt**GAAA**gatggcatca
Vibrio	ataaccattg**GAAA**cgatggctaa	---
B. cereus	ataactccgg**GAAA**ccggggctaa	cgcatggttc**GAAA**tt**GAAA**ggcg
C. botulinum	atagccttcc**GAAA**ggaagattaa	---
Campylobacter	acaacagttg**GAAA**cgactgctaa	gttgagtagg**GAAA**gtttttcggt
Yersinia	ataactactg**GAAA**cggtagctaa	---
Listeria	ataactccgg**GAAA**ccggggctaa	ccacgctttt**GAAA**gatggtttcg

Region in E.coli 16S rRNA	370-400	485-515
E.coli	---	---
Salmonella	---	---
Staphylococcus	cgcaatgggc**GAAA**gcctgacgga	tacctaatca**GAAA**gccacggcta
C. perfringens	agggtcattg**GAAA**ctg**GAAA**act	---
Vibrio	---	---
B. cereus	cgcaatggac**GAAA**gtctgacgga	tacctaacca**GAAA**gccacggcta
C. botulinum	cgcaatgggg**GAAA**ccctgacgga	---
Campylobacter	cgcaatgggg**GAAA**ccctgacgca	---
Yersinia	---	---
Listeria	cgcaatggac**GAAA**gtctgacgga	tatctaacca**GAAA**gccacggcta

Region in E.coli 16S rRNA	595-625	625-655
E.coli	agtcagatgt**GAAA**tccccgggct	---
Salmonella	agtcggatgt**GAAA**tccccgggct	aactgcattc**GAAA**ctggcaggct
Staphylococcus	agtctgatgt**GAAA**gcccacggct	agggtcattg**GAAA**ctg**GAAA**act
C. perfringens	agtgggatgt**GAAA**tacccgggct	---
Vibrio	agtcagatgt**GAAA**gcccggggct	nattgcattt**GAAA**ctggcagact
B. cereus	agtctgatgt**GAAA**gcccacggct	agggtcattg**GAAA**ctgggagact
C. botulinum	agtgggatgt**GAAA**tccccgggct	---
Campylobacter	agtctcttgt**GAAA**tctaatggct	aactgcttgg**GAAA**ctgatagtct
Yersinia	cagtcagatgt**GAAA**tccccgcgct	aactgcattt**GAAA**ctggcaagct
Listeria	agtctgatgt**GAAA**gcccccggct	agggtcattg**GAAA**ctggaagact

Region in E.coli 16S rRNA	650-680	660-690
E.coli	---	---
Salmonella	---	---
Staphylococcus	ttg**GAAA**ctg**GAAA**acttgagtgc	tgcagaagag**GAAA**gtggaattcc
C. perfringens	---	---
Vibrio	---	---
B. cereus	---	tgcagaagag**GAAA**gtggaattcc
C. botulinum	---	tgcaggagag**GAAA**gcggaattcc
Campylobacter	---	---
Yersinia	---	---
Listeria	---	---

Region in E.coli 16S rRNA	685-715	755-780
E.coli	gtgtagcggt**GAAA**tgcgtagaga	gctcaggtgc**GAAA**gcgtggggag
Salmonella	gtgtagcggt**GAAA**tgcgtagaga	gctcaggtgc**GAAA**gcgtggggag
Staphylococcus	gtgtagcggt**GAAA**tgcgcagaga	gctgatgtgc**GAAA**gcgtggggat
C. perfringens	gtgtagcggt**GAAA**tgcgtagaga	gctgaggctc**GAAA**gcgtggggag
Vibrio	gtgtagcggt**GAAA**tgcgtagaga	---
B. cereus	gtgtagcggt**GAAA**tgcgtagaga	actgaggcgc**GAAA**gcgtggggag
C. botulinum	gtgtagcggt**GAAA**tgcgtagaga	gctgaggcac**GAAA**gcgtgggtag
Campylobacter	---	gctaaggcgc**GAAA**gcgtggggag
Yersinia	gtgtagcggt**GAAA**tgcgtagaga	gctcaggtgc**GAAA**gcgtggggag
Listeria	gtgtagcggt**GAAA**tgcgtagata	gctgaggcgc**GAAA**gcgtggggag

Region in E.coli 16S rRNA	895-925	1000-1050
E.coli	---	---
Salmonella	---	---
Staphylococcus	ccgcaaggtt**GAAA**ctcaaaggaa	---
C. perfringens	---	ctttaatcgag**GAAA**tccttcgggg
Vibrio	---	---
B. cereus	ccgcaaggct**GAAA**ctcaaaggaa	---
C. botulinum	---	---
Campylobacter	---	---
Yersinia	---	---
Listeria	ccgcaaggtt**GAAA**ctcaaaggaa	---

Region in E.coli 16S rRNA	1065-1095	1245-1275
E.coli	ctcgtgttgt**GAAA**tgttgggtta	---
Salmonella	ctcgtgttgt**GAAA**tgtcgggtta	---
Staphylococcus	---	aaagggcagc**GAAA**ccgcgaggtc
C. perfringens	---	---
Vibrio	ctcgtgttgt**GAAA**tgttgggtta	gccaacttgc**GAAA**gtgagcgaat
B. cereus	---	---
C. botulinum	---	---
Campylobacter	---	---
Yersinia	ctcgtgttgt**GAAA**tgttgggtta	---
Listeria	---	---

Region in E.coli 16S rRNA	1305-1335
E.coli	---
Salmonella	---
Staphylococcus	---
C. perfringens	attgtaggct**GAAA**ctcgcctaca
Vibrio	---
B. cereus	---
C. botulinum	---
Campylobacter	---
Yersinia	---
Listeria	---

Table II: GAAA in 16S rRNA

Region in E.coli 16S rRNA	70-100	115-145
S. aureus	---	---
S. epidermidis	---	---
S. pneumoniae	---	---
S. pyogenes	---	---
E. faecalis	cactcaattg**GAAA**gaggagtggc	---
N. meningitidis	---	---
E. coli	---	taatgtctgg**GAAA**ctgcctgatg
Enterobacter spec.	---	taatgtctgg**GAAA**ctgccgatgg
Proteus spec.	---	ggtaacagga**GAAA**gcttgctttc
P. aeruginosa	---	---
P. fluorescens	---	---
P. mendocina	---	---
P. syringae	---	---
H. influenzae	---	ggtagcagga**GAAA**gcttgctttc
H. ducreyi	---	---
Bacteroides spec.	---	---

Region in E.coli 16S rRNA	145-175	180-210
S. aureus	ataacttcgg**GAAA**ccggagctaa	gttcaaaagt**GAAA**gacggtcttg
S. epidermidis	ataacttcgg**GAAA**ccggagctaa	gttcaatagt**GAAA**gacggttttg
S. pneumoniae	ataactattg**GAAA**cgatagctaa	---
S. pyogenes	ataactattg**GAAA**cgatagctaa	---
E. faecalis	ataacacttg**GAAA**caggtgctaa	gcataagagt**GAAA**ggcgctttcg
N. meningitidis	ataactgatc**GAAA**gatcagctaa	tcttgagaga**GAAA**gcaggggacc
E. coli	ataactactg**GAAA**cggtagctaa	---
Enterobacter spec.	ataactactg**GAAA**cggtagctaa	---
Proteus spec.	ataactactg**GAAA**cggtggctaa	---
P. aeruginosa	ataacgtccg**GAAA**cggccgctaa	tcctgaggga**GAAA**gtcggggatc
P. fluorescens	ataacgttcg**GAAA**cggacgctaa	tcctacggga**GAAA**gcagggacc
P. mendocina	ataacgttcc**GAAA**ggaacgctaa	tcctacggga**GAAA**gcangggacc
P. syringae	ataacgctcg**GAAA**cggacgctaa	tcctacggga**GAAA**gcaggggacc
H. influenzae	ataactactg**GAAA**cggtagctaa	taaaggggc**GAAA**gctgttgcca
H. ducreyi	ataactacgg**GAAA**ctgtagctaa	---
Bacteroides spec.	atagcctttc**GAAAGAAA**gattaa	---

Region in E.coli 16S rRNA	370-400	450-480
S. aureus	cgcaatgggc**GAAA**gcctgacgga	---
S. epidermidis	cgcaatgggc**GAAA**gcctgacgga	---
S. pneumoniae	---	tgtgagagtg**GAAA**gttcacactg
S. pyogenes	---	ggtgggagtg**GAAA**atccaccaag
E. faecalis	ggcaatggac**GAAA**gtctgaccga	---
N. meningitidis	---	tgtcagggaa**GAAA**aggctgttgc
E. coli	---	---
Enterobacter spec.	---	---
Proteus spec.	---	---
P. aeruginosa	---	---
P. fluorescens	gacaatgggc**GAAA**gcctgatcca	---
P. mendocina	gacaatgggc**GAAA**gcctnatcca	---
P. syringae	gacaatgggc**GAAA**gcctgatcca	---
H. influenzae	cgcaatgggg**GAAA**ccctgatgca	---
H. ducreyi	cacaatgggg**GAAA**ccctgatgca	---
Bacteroides spec.	---	---

Region in E.coli 16S rRNA	485-515	595-625
S. aureus	tacctaatca**GAAA**gccacggcta	agtctgatgt**GAAA**gcccacggct
S. epidermidis	tacctaatca**GAAA**gccacggcta	agtctgatgt**GAAA**gcccacggct
S. pneumoniae	tatcttacca**GAAA**gggacggcta	---
S. pyogenes	taactaacca**GAAA**gggacggcta	---
E. faecalis	tatctaacca**GAAA**gccacggcta	agtctgatgt**GAAA**gcccccggct
N. meningitidis	---	agcaggatgt**GAAA**tccccgggct
E. coli	---	agtcagatgt**GAAA**tccccgggct
Enterobacter spec.	---	aagtcgatgt**GAAA**tccccgggct
Proteus spec.	---	agtcagatgt**GAAA**gccccgagct
P. aeruginosa	---	agcttgatgt**GAAA**tccccgggct
P. fluorescens	---	agttggatgt**GAAA**tccccgggct
P. mendocina	---	agttggatgt**GAAA**gccccgggct
P. syringae	---	agttgaatgt**GAAA**tccccgggct
H. influenzae	---	agtgaggtgt**GAAA**gccctgggct
H. ducreyi	---	agtgagatgt**GAAA**gccccgggct
Bacteroides spec.	---	agtcagttgt**GAAA**gtttgcggct

Region in E.coli 16S rRNA	625-655	650-680
S. aureus	agggtcattg**GAAA**ctg**GAAA**act	ttg**GAAA**ctg**GAAA**acttgagtgc
S. epidermidis	agggtcattg**GAAA**ctg**GAAA**act	ttg**GAAA**ctg**GAAA**acttgagtgc
S. pneumoniae	gtaggctttg**GAAA**ctgtttaact	---
S. pyogenes	gtacgctttg**GAAA**ctggagaact	---
E. faecalis	agggtcattg**GAAA**ctgggagact	---
N. meningitidis	---	---
E. coli	---	---
Enterobacter spec.	aactgcattg**GAAA**ctggcagctt	---
Proteus spec.	aactgcatct**GAAA**ctggctggct	---
P. aeruginosa	---	---
P. fluorescens	---	---
P. mendocina	---	---
P. syringae	---	---
H. influenzae	---	---
H. ducreyi	---	---
Bacteroides spec.	aattgcagtt**GAAA**ctggcagtct	---

Region in E.coli 16S rRNA	660-690	685-715
S. aureus	tgcagaagag**GAAA**gtggaattcc	gtgtagcggt**GAAA**tgcgcagaga
S. epidermidis	tgcagaagag**GAAA**gtggaattcc	gtgtagcggt**GAAA**tgcgcagaga
S. pneumoniae	---	gtgtagcggt**GAAA**tgcgtagata
S. pyogenes	---	gtgtagcggt**GAAA**tgcgtagata
E. faecalis	---	gtgtagcggt**GAAA**tgcgtagata
N. meningitidis	---	gtgtagcagt**GAAA**tgcgtagaga
E. coli	---	gtgtagcggt**GAAA**tgcgtagaga
Enterobacter spec.	---	gtgtagcggt**GAAA**tgcgtagaga
Proteus spec.	---	gtgtagcggt**GAAA**tgcgtagaga
P. aeruginosa	---	gtgtagcggt**GAAA**tgcgtagata
P. fluorescens	---	gtgtagyggt**GAAA**tgcgttgata
P. mendocina	---	gtgtagcggt**GAAA**tgcgtagata
P. syringae	---	gtgtagcggt**GAAA**tgcgtagata
H. influenzae	---	gtgtagcggt**GAAA**tgcgtagaga
H. ducreyi	---	gtgtagcggt**GAAA**tgcgtagaga
Bacteroides spec.	---	gtgtagcggt**GAAA**tgcttagata

Region in E.coli 16S rRNA	715-745	755-780
S. aureus	---	gctgatgtgc**GAAA**gcgtggggat
S. epidermidis	---	gctgatgtgc**GAAA**gcgtggggat
S. pneumoniae	caccggtggc**GAAA**gcggctctct	gctgaggctc**GAAA**gcgtggggag
S. pyogenes	caccggtggc**GAAA**gcggctctct	gctgaggctc**GAAA**gcgtggggag
E. faecalis	---	gttcatgccc**GAAA**gcgtgggtag
N. meningitidis	---	gctcaggtgc**GAAA**gcgtggggag
E. coli	---	gctcaggtgc**GAAA**gcgtggggag
Enterobacter spec.	---	gctcaggtgc**GAAA**gcgtggggac
Proteus spec.	---	actgaggtgc**GAAA**gcgtggggag
P. aeruginosa	---	actgaggtgc**GAAA**gcgtggggag
P. fluorescens	---	actgaggtgc**GAAA**gcgtggggag
P. mendocina	---	actgaggtgc**GAAA**gcgtggggag
P. syringae	---	gctcatgtgt**GAAA**gcgtggggag
H. influenzae	---	gctcatgtgc**GAAA**gcgtggggag
H. ducreyi	---	actgatgctc**GAAA**gtgtgggtat
Bacteroides spec.	---	

Region in E.coli 16S rRNA	845-475	895-925
S. aureus	---	ccgcaaggtt**GAAA**ctcaaaggaa
S. epidermidis	---	ccgcaaggtt**GAAA**ctcaaaggaa
S. pneumoniae	---	ccgcaaggtt**GAAA**ctcaaaggaa
S. pyogenes	---	ccgcaaggtt**GAAA**ctcaaaggaa
E. faecalis	---	ccgcaaggtt**GAAA**ctcaaaggaa
N. meningitidis	gctaacgcgt**GAAA**ttgaccgcct	---
E. coli	---	---
Enterobacter spec.	---	---
Proteus spec.	---	---
P. aeruginosa	---	---
P. fluorescens	---	---
P. mendocina	---	---
P. syringae	---	---
H. influenzae	---	---
H. ducreyi	---	---
Bacteroides spec.	---	cggcaacggt**GAAA**ctcaaaggaa

Region in E.coli 16S rRNA	1065-1095	1245-1275
S. aureus	---	aaagggcagc**GAAA**ccgcgaggtc
S. epidermidis	---	aaagggtagc**GAAA**ccgcgaggtc
S. pneumoniae	---	---
S. pyogenes	---	---
E. faecalis	---	---
N. meningitidis	---	---
E. coli	ctcgtgttgt**GAAA**tgttgggtta	---
Enterobacter spec.	ctcgtgttgt**GAAA**tgttgggtta	---
Proteus spec.	tcgttgttgt**GAAA**tgttgggtta	---
P. aeruginosa	---	---
P. fluorescens	---	---
P. mendocina	---	---
P. syringae	---	---
H. influenzae	ctcgtgttgt**GAAA**tgttgggttn	gcgaatctca**GAAA**gtgcatctaa
H. ducreyi	ctcgtgttgt**GAAA**tgttgggttn	---
Bacteroides spec.	---	---

Region in E.coli 16S rRNA	1400-1430
S. aureus	---
S. epidermidis	---
S. pneumoniae	---
S. pyogenes	---
E. faecalis	---
N. meningitidis	---
E. coli	---
Enterobacter spec.	---
Proteus spec.	---
P. aeruginosa	---
P. fluorescens	---
P. mendocina	---
P. syringae	---
H. influenzae	---
H. ducreyi	---
Bacteroides spec.	gaataacgtg**GAAA**catgttagcc

The invention is explained in more detail in the following using examples and figures.

Description of the figures:

Fig. 1: General scheme of NASBA® combined with ribozymes for real-time detection.
Ribozyme motif within one of the two primers. Only one possibility is shown in which the ribozyme motif is located at the 3' end of the amplified RNA. The RNA substrate probe is marked with a fluorescence dye, the reporter (circle) and a quencher (triangle). In the intact probe, the efficient interaction of both labels leads to "FRET" or quenching, i.e. to no (or only a very weak) reporter signal (empty circle). The ribozyme cleaves many probe molecules. In the cleaved probe, both labels are separated and a strong reporter signal is produced (filled circles).

Fig. 2: A: General structure of hammerhead ribozymes. Only preserved nucleotides are identified by corresponding letters, all non-preserved positions are shown as N. The length of the hybridizing arms can be adjusted to the requirements in each case. Three locations for possible hairpin loops are shown by dotted lines. The polarity (5'-3' direction) is given only for the cleaved section. B: Corresponds to Fig. 2A, the positions at which the ribonucleotides are preferably used being provided with the prefix "r", while the remaining nucleotides can in each case be either ribo- or deoxyribonucleotides.

Fig. 3: A possibility for cleaving a minimal ribozyme and a nucleic acid substrate probe. The preserved ribozyme motif was shortened to GAAA.

Fig. 4: A: Based on the possibility shown in Fig. 3, an amplified nucleic acid (thick line) is shown with the minimal ribozyme motif. The nucleic acid substrate probe contains reporters and quenchers (a few possibilities are given below) at both ends, but they can also be linked to other positions. B: Corresponds to Fig. 4A, the positions at which ribonucleotides are preferably placed are provided with the prefix "r", while the remaining nucleotides can in each case be either ribo- or deoxyribonucleotides.

Fig. 5: A further possibility for cleaving a nucleic acid substrate probe. The preserved ribozyme motif is reduced to CUGA-N-GA.

Fig. 6: Based on the possibility shown in Fig. 5, an amplified nucleic acid (thick line) is shown with the minimal ribozyme motif. The nucleic acid substrate probe contains reporters and quenchers at both ends, but they can also be linked to other positions (cf. Fig. 4).

Fig. 7: Based on the possibility shown in Fig. 3, the reverse primer contains the ribozyme motif. The hybrid between primary target nucleic acid and primer is shown above. The position within the target nucleic acid and the length of the base-pair-forming section can vary. The resulting amplified nucleic acid with the complete ribozyme motif is shown below.

Fig. 8: Based on the possibility shown in Fig. 3, the reverse primer contains the ribozyme motif in a bulge. The hybrid between primary target nucleic acid and primer is shown above. The position within the target nucleic acid and the length of both base-pair-forming sections can vary. The resulting amplified nucleic acid with the complete ribozyme motif is shown below.

Fig. 9: Based on the possibility shown in Fig. 3, the reverse primer contains the ribozyme motif in a bulge, followed by a very short 3'-terminal base-paired section. As is shown, this section can overlap with the ribozyme motif and the bulge can be so short that it comprises only one nucleotide. The hybrid between primary target nucleic acid and primer is shown above. The position within the target nucleic acid and the length of both base-pair-forming sections can vary. The resulting amplified nucleic acid with the complete ribozyme motif is shown below.

Fig. 10: Based on the possibility shown in Fig. 2B, the reverse primer contains the ribozyme motif in a bulge followed by a single rA-T base pairing with the target sequence. The hybrid between primary target nucleic acid and primer is shown above. The position within the target nucleic acid and the length of both base-pair-forming sections can vary. The resulting amplified nucleic acid with the complete ribozyme motif is shown below.

Fig. 11: Corresponds to the possibility shown in Fig. 10. Here, however, the target sequence already contains a longer stretch of the ribozyme motif (or, as shown, of the complete motif).

Fig. 12: By way of example, structure of a DNAzyme(= catalytic DNA). The substrate can either be wholly RNA, or a minimum of rA must be present.

Fig. 13: By way of example, structure of another DNAzyme. The substrate can either be wholly RNA or a minimum of rRrY must be present.

Fig. 14: Corresponds to Fig. 10, the primer containing the greatest part of the Nazyme motif (of the catalytic nucleic acid motif) and only the two last nucleotides being absent. Shown here is a possibility based on "prototype A". For "prototype B", the presence of longer motifs (e.g. TCGTTG instead of TCGT) makes it possible to use a more deleted motif in the primer, the 3'-terminal ACGA in the elongated primer being supplied by the target sequence.

Fig. 15: Example of a universal ribozyme probe.

Fig. 16: Example of a HIV ribozyme probe.

EXAMPLES

Material:

The primers and probes used within the framework of the invention can be obtained by the route familiar to a person skilled in the art, such as e.g. by oligonucleotide synthesis.

Example 1

NASBA® reaction in combination with ribozyme-dependent detection:

All enzymes were commercially available from Pharmacia, with the exception of AMV reverse transcriptase, which was obtained from Seikagaku.

23µl NASBA® reaction mixture, including 5 µl from the purification according to Boom et al. (J. Clin. Microbiol. 28 (1990) 495-503) (final

concentration in 25 µl reaction mixture: 40 mM Tris, pH 8.5, 12 mM $MgCl_2$, 42 mM KCl, 15 v/v DMSO, 1 mM each dNTP, 2 mM each NTP, 0.2 µM primer 1, 0.2 µM primer 2 and 0.1-0.5 µM substrate probe) were incubated at 65°C for 5 minutes to make possible a destabilization of the secondary structures in the RNA. This was followed by cooling to 41°C for the primer annealing. The amplification was started by the addition of 2 µl enzyme mixture (0.1 µg/µl BSA, 0.1 units RNase H, 40 units t7 RNA polymerase and 8 units AMV reverse transcriptase). The reaction was incubated at 41°C for 90 minutes. During the reaction, the fluorescence signals were measured in the ABI Prism 7700 sequence detector. The combination FAM/RAMRA was used as reporter/quencher.

<u>Experiment A:</u>

(dNTP = upper case letters: rNTP = lower case letters)

Primer 1:	5'-AAT TCT AAT ACG ACT CAC TAT AGG GTG CTA TGT CAC TTC CCC TTG GTT CTC TCA-3'
Primer 2:	5'-GAA TCT CAT CAG TAG CGA GTG GGG GGA CAT CAA GCA GCC ATG CAA A-3'
Substrate A:	5'-TAMRA-Tga auc gaa acg cga aag cgu cua gcg u-FAM-3'

<u>Experiment B:</u>

Primer 1:	5'-AAT TCT AAT ACG ACT CAC TAT AGG GTG CTA TGT CAC TTC CCC TTG GTT CTC TCA-3'
Primer 2:	5'-ACG TAG TTT CGG CCT TTC GGC CTC ATC AGC GTG CAG TGG GGG GAC ATC AAG CAG CCA TGC AAA-3'
Substrate B:	5'-TAMRA-Tac gua guc cgu gcu-FAM-3'

Quantification:

For the quantitative determination of the HIV-RNA, 4 external controls and 2 unknown samples as well as 2 negative controls were introduced into the amplification described above. By means of the standard, a standard curve was produced, and the concentration of the standard was:

Q1 approx. 1,000,000 molecules (RNA)
Q2 approx. 100,000 molecules (RNA)
Q3 approx. 10,000 molecules (RNA)
Q4 approx. 1,000 molecules (RNA)

Experiments A and B led to the following result: the fluorescence, measured in the ABI PRISM 7700, of the reporter dye FAM increased according to the quantity of target molecule (RNA) used. It was shown that after t=15 minutes, with the highest standard molecule quantity used, the threshold value for a defined positive signal was reached (5x std. dev. of the background). The other standards reached the corresponding threshold value after t= 20, 24 and 26 minutes. The unknown samples reached their threshold value after approx. t=18 and t=23 minutes. Using the standard curve established by means of the standards, a molecule quantity of approx. 200,000 (t=18) and 15,000 (t=23) respectively resulted for the unknown samples. The negative controls did not reach the threshold value. This shows that a quantification of target molecules is possible using the technique described here.

Example 2

Universal recognition of any (full-size) amplified RNA targets (ribozyme motifs in reverse primer). The corresponding "universal ribozyme probe" was added to the NASBA[3] amplification kit.

At its 3′ end, the reverse primer contains the usual target-specific sequence (N) and in addition at its 5′ end a sequence which codes for the general universal ribozyme motif: 5′-GCG TTT CGA TTC CHH NNN N..

The transcript ends with the sequence
5′-...N NNN NHG GAA UCG AAA CGC

The ribozyme probe contained the following sequence:
5′-GCG UC – U AGC GGA AAC GCU ACU GAX GAG AUU CC (32-mer)
– cleavage site

Two dyes 5′-Q and 3′-R (or 3′-Q and 5′-R) were linked to the ends.

For the quantitative determination of HIV-RNA, 4 external controls and 2 unknown samples as well as 2 negative controls were introduced into the amplification described above. By means of the standard, a standard curve was produced, and the concentration of the standard was:

Q1 approx. 1,000,000 molecules (RNA)
Q2 approx. 100,000 molecules (RNA)
Q3 approx. 10,000 molecules (RNA)
Q4 approx. 1,000 molecules (RNA)

The experiment in example 2 led to the following result: the fluorescence, measured in the ABI PRISM 7700, of the reporter dye FAM increased according to the quantity of target molecule (RNA) used. It was shown that after t=12 minutes, with the highest standard molecule quantity used, the threshold value for a defined positive signal was reached (5x std. dev. of the background). The other standards reached the corresponding threshold value after t= 18, 22 and 25 minutes. The unknown

samples reached their threshold value after approx. t=18 and t=23 minutes. Using the standard curve established by means of the standards, a molecule quantity of approx. 100,000 (t=18) and 9,000 (t=23) respectively resulted for the unknown samples. The negative controls did not reach the threshold value. This shows that a quantification of target molecules is possible using the technique described here.

This example probe can by lengthened at one or both ends by more base-paired nucleotides.

Example 3

Specific recognition of an amplified target sequence: proximal to one of the primers.

The present specific example carried out by means of an NASBA® supported detection of HIV (corresponding to USP 5,837,501).

Amplified segment of the HIV-RNA:

agtgggggacatcaagcagctatgcaaa(c,t)gttaaaagatactatcaatgaggaagctgcagaatgggacagggtacatccagtacatgcagggcctattccaccaggccagatgagagaaccaaggggaagtgacatagca

(only one strand is shown, the primer sequences are underlined). The proximal sequence is likewise highly preserved and includes the following section:

agcagctatgGaaa(c,t)gttaaaaga

The forward primer for the introduction of the T7 promoter sequence (upper case letters) and 1 point mutation (bold upper case letters):

AATTCTAATACGACTCACTATAGGG**agtgggggacatcaagcagctatgGaaa**

The transcript product contains the GAAA ribozyme motif which is linked to the proximal HIV-specific sequence:
GGGagcagctatgGaaa(c,t) gttaaaaga...

The process can be carried out in particular with the complementary ribozyme probe corresponding to the general test protocol.

For the quantitative determination of the HIV-RNA, 4 external controls and 2 unknown samples as well as 2 negative controls were introduced into the amplification described above. By means of the standard, a standard curve was produced, and the concentration of the standard was:

Q1 approx. 1,000,000 molecules (RNA)
Q2 approx. 100,000 molecules (RNA)
Q3 approx. 10,000 molecules (RNA)
Q4 approx. 1,000 molecules (RNA)

The experiment in example 3 led to the following result: the fluorescence, measured in the ABI PRISM 7700, of the reporter dye FAM increased according to the quantity of target molecule (RNA) used. It was shown that after t=22 minutes, with the highest standard molecule quantity used, the threshold value for a defined positive signal was reached (5x std. dev. of the background). The other standards reached the corresponding threshold value after t= 24, 28 and 33 minutes. The unknown samples reached their threshold value after approx. t=18 and t=23 minutes. Using the standard curve established by means of the standards, a molecule quantity of approx. 400,000 (t=23) and 10,000 (t=28) respectively resulted for the unknown samples. The negative controls did not reach the threshold value. This shows that a quantification of target molecules is possible using the technique described here.

Example 4

A. GAAA in rRNA sections for the specific detection of bacteria species.

The most important pathogens transmitted by foodstuffs are listed in the tables above.

Unique sequence motifs (shaded) lie between positions 110 and 700 (according to E. coli numbering system). Highly preserved primers are known for 16S rRNA amplification: 110 f and 700r [Lane, D.J. (1991). 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematics, E. Stackebrandt and M. Goodfellow, Eds. (New York: Willey), pp. 115-175].

B. Specific detection of sepsis pathogens.

In the above tables, the most important sepsis pathogens are also listed.

Unique sequence motifs (shaded) which can be used according to the invention lie between positions 110 and 530 (according to E. coli numbering system).
Highly preserved primers for 16S rRNA amplification are known: [Lane, D.J. (1991). 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematics, E. Stackebrandt and M. Goodfellow, eds. (New York: Willey), pp. 115-175].

The sequence motifs contained in the 16S rRNA can be used for the processes according the invention so that processes for the detection of pathogens, in particular of sepsis pathogens and foodstuff germs, and kits provided for same are also made available within the framework of the present invention.

Tab. III: Dyes suitable as reporters/quenchers

Tab. III (2nd continuation)

A (nm)	E (nm)	Dyes
349	448	AMCA
336	490	ADANS
495	503	BODIPY 493/503
505	513	BODIPY LF
494	515	6-FAM, Fluorescein
496	516	6-OREGON Green 488
521	536	TET
518	543	Rhodamine 6G (6-R6G)
531	545	BODIPY FL Br2
528	547	BODIPY R6G
527	548	6-JOE
535	552	BODIPY 530/550
535	555	HEX
552	565	Cy3
559	569	BODIPY 558/568
542	574	BODIPY TMR 542/574
546	579	5-TAMRA
560	580	NED
575	602	6-ROX
583	603	TEXAS Red
588	616	BODIPY TR 589/617
630	640	Light Cycler RED 640
625	640	BODIPY 630/650
646	660	BODIPY 650/665
651	674	Cy5
700	710	Light Cycler Red
678	703	Cy 5.5
685	705	IRD 700
685	705	La Jolla Blue
743	767	Cy 7
787	807	IRD 41

Patent claims:

1. Process for the amplification and quantitative real-time detection of nucleic acids, **characterized in that**

   a) a primer is used to which a nucleic acid sequence, preferably with a length of 1 to 40 nucleotides, is attached, which codes for the sequence motif 5'-GAAA-3' (motif A) in the transcript,

   b) the amplification being carried out in the presence of an excess, preferably in a concentration of 50 to 500 nM, of a nucleic acid probe, preferably with a length of 25 to 60 nucleotides (particularly preferably approx. 50 nucleotides) which contains the sequence motif 5'-CUGANGA-3' (motif B), a reporter molecule and a quencher molecule being attached to each probe molecule, and

   c) the original concentration of the nucleic acid in the sample is determined by measuring the time-dependent change in fluorescence during amplification, the relative concentration "$C_{rel.}$"

being determined according to the following formula:

$$C_{rel.}=t_{i}/t_{Ref.}$$

where

t_{E} corresponds to the time measured for the sample from the start of amplification to the reaching of the fluorescence threshold value and

$t_{Ref.}$ corresponds to time measured for a reference nucleic acid of known concentration from the start of amplification to the reaching of the fluorescence threshold value.

2. Process for the amplification and quantitative real-time detection of nucleic acids, **characterized in that**

a) a primer is used to which a nucleic acid sequence, preferably with a length of 1 to 40 nucleotides, is attached, which codes for the sequence motif 5'-CUGANGA-3' (motif B) in the transcript,

b) the amplification is carried out in the presence of an excess, preferably in a concentration of 50 to 500 nM, of a nucleic acid probe, preferably with a length of 25 to 60 nucleotides (particularly preferably approx. 50 nucleotides) which contains the sequence motif 5'-GAAA-3' (motif A), a reporter molecule and a quencher molecule being attached to each probe molecule, and

c) the original concentration of the nucleic acid in the sample is determined by measuring the time-dependent change in fluorescence during the amplification, the relative concentration "$C_{r\cdot l.}$" being determined according to the following formula:

$$C_{rel.}=t_{r}/t_{Ref.}$$

where

t_P corresponds to the time measured for the sample from the start of the amplification to the reaching of the fluorescence threshold value and

$t_{Ref.}$ corresponds to time measured for a reference nucleic acid of known concentration from the start of the amplification to the reaching of the fluorescence threshold value.

3. Process for the amplification and quantitative real-time detection of a nucleic acid containing the sequence motif 5'-GAAA-3' (motif A), **characterized in that**

   a) the sequences of the primers used are chosen such that the sequence range of the nucleic acid which contains motif A is amplified,

   b) the amplification being carried out in the presence of an excess of a nucleic acid probe which contains the sequence motif 5'-CUGANGA-3' (motif B), a reporter molecule and a quencher molecule being attached to each probe molecule, and

   c) the original concentration of the nucleic acid in the sample is determined by measuring the time-dependent change in fluorescence during the amplification, the relative concentration "$C_{rel.}$" being determined according to the following formula:

$$C_{rel.}=t_P/t_{Ref.}$$

   where
   t_P corresponds to the time measured for the sample from the start of the amplification to the reaching of the fluorescence threshold value and

   $t_{Ref.}$ corresponds to the time measured for a reference nucleic acid of known concentration from the start of the amplification to the reaching of the fluorescence threshold value.

4. Process for the amplification and quantitative detection of a nucleic acid containing the sequence motif 5'-CUGANGA-3' (motif B), **characterized in that**

a) the sequences of the primers used are chosen such that the sequence range of the nucleic acid which contains motif B is amplified,

b) the amplification being carried out in the presence of an excess of a nucleic acid probe which contains the sequence motif 5'-GAAA-3' (motif A), a reporter molecule and a quencher molecule being attached to each probe molecule, and

c) the original concentration of the nucleic acid in the sample is determined by measuring the time-dependent change in fluorescence during the amplification, the relative concentration "$C_{r-l.}$" being determined according to the following formula:

$$C_{rel.}=t_{t}/t_{Ref.}$$

where

t_p corresponds to the time measured for the sample from the start of the amplification to the reaching of the fluorescence threshold value and

$t_{Ref.}$ corresponds to the time measured for a reference RNA of known concentration from the start of the amplification to the reaching of the fluorescence threshold value.

5. Process according to claim 1 , **characterized in that** the nucleic acid is RNA, DNA or a DNA/RNA chimera.

6. Process according to claim 1 , **characterized in that** the nucleic acid sequence attached to the primer has a length of 1 to 40 nucleotides.

7. Process according to claim 1 , **characterized in that** the nucleic acid probe is used in a concentration of 50 to 500 nM.

8. Process according to claim 1 , characterized in that the nucleic acid probe has a length of 25 to 60 nucleotides, preferably approx. 50 nucleotides.

9. Process according to claim 1 , characterized in that the amplification process is an isothermal or cyclical amplification process.

10. Process according to claim 9, **characterized in that** the amplification process is selected from the group consisting of NASBA®, TMA, 3SR or PCR.

11. Process according to claim 1 , **characterized in that** there is used, as reporter, a dye from the group consisting of FAM, HEX, TET, ALEXA, Texas Red, Light Cycler Red, IRD 700, CY-7, IRD 41 or La Jolla Blue and, as quencher, a dye from the group consisting of TAMRA, CY-5,DABCYL and LCR.

12. Process for the detection of bacterial pathogens in a sample, **characterized in that** the sample is brought into contact with a probe which contains the sequence motif 5'-CUGANGA-3' (motif B), a reporter molecule and a quencher molecule being attached to each sequence motif, the probe containing a sequence suitable for the hybridization with a section of the 16S rRNA of the pathogens containing the sequence motif 5'-GAAA-3' (motif A), and the pathogens being detected by measuring the occurring fluorescence signal.

13. Process according to claim 12, **characterized in that** the pathogens are selected from the group consisting of E. coli, Salmonella, Staphylococcus, C. perfringens, Vibrio, B. cereus, C. botulinum, Campylobacter, Yersinia and Listeria.

14. Process according to claim 12, **characterized in that** the nucleic acid probe has a length of 25 to 60 nucleotides, preferably approx. 50 nucleotides.

15. Process according to claim 12 , **characterized in that** there is used as reporter, a dye from the group consisting of FAM, HEX, TET, ALEXA, Texas Red, Light Cycler Red, IRD 700, CY-

7, IRD 41 or La Jolla Blue and, as quencher, a dye from the group consisting of TAMRA, CY-5, DABCYL and LCR.

16. Kit for carrying out the process according to claim 1, **characterized in that** it comprises

a) an amplification primer to which a nucleic acid sequence is attached, which codes for the sequence motif 5'-GAAA-3' in the transcript,
b) a further amplification primer,
c) enzymes and reagents for carrying out the amplification,
d) a nucleic acid probe which contains the sequence motif 5'-CUGANGA-3', a reporter molecule and a quencher molecule being attached to each probe molecule, as well as optionally
e) apparatus and auxiliaries necessary for carrying out the reaction.

17. Kit for carrying out the process according to claim 2, **characterized in that** it comprises

a) an amplification primer to which a nucleic acid sequence is attached, which codes for the sequence motif 5'-CUGANGA-3' in the transcript,
b) a further amplification primer,
c) enzymes and reagents for carrying out the amplification,
d) a nucleic acid probe which contains the sequence motif 5'-GAAA-3', a reporter molecule and a quencher molecule being attached to each probe molecule, as well as optionally
e) apparatus and auxiliaries necessary for carrying out the reaction.

18. Kit for carrying out the process according to claim 3, **characterized in that** it comprises

a) two amplification primers,
b) enzymes for carrying out the amplification,
c) a nucleic acid probe which contains the sequence motif 5'-CUGANGA-3', a reporter molecule and a quencher

molecule being attached to each probe molecule, as well as optionally

d) apparatus and auxiliaries necessary for carrying out the reaction.

19. Kit for carrying out the process according to claim 4, **characterized in that** it comprises

a) two amplification primers,

b) enzymes for carrying out the amplification,

c) a nucleic acid probe which contains the sequence motif 5'-GAAA-3', a reporter molecule and a quencher molecule being attached to each probe molecule, as well as optionally

d) apparatus and auxiliaries necessary for carrying out the reaction.

20. Kit according to claim 16 , characterized in that the nucleic acid is RNA, DNA or a DNA/RNA chimera.

21. Kit according to claim 16 , **characterized in that** the nucleic acid sequence attached to the primer has a length of 1 to 40 nucleotides.

22. Kit according to claim 16 , **characterized in that** the nucleic acid probe is used in a concentration of 50 to 500 nM.

23. Kit according to claim 16 , **characterized in that** the nucleic acid probe has a length of 25 to 60 nucleotides, preferably approx. 50 nucleotides.

24. Kit according to claim 16 , **characterized in that** the amplification process is an isothermal or cyclical amplification process.

25. Kit according to claim 24, **characterized in that** the amplification process is selected from the group consisting of NASBA, TMA, 3SR or PCR.

26. Kit according to claim 25, **characterized in that** it is a kit for carrying out a NASBA®, the enzymes displaying the activity of reverse transcriptase, T7 RNA polymerase and RNase H.

27. Kit according to claim 26, **characterized in that** the enzymes for carrying out the NASBA® are reverse transcriptase, T7 RNA polymerase and RNAse H.

28. Kit for carrying out the process according to one of claim 12 , **characterized in that** it comprises a probe with a sequence suitable for the hybridization with a section of the 16S rRNA of the pathogens containing the sequence motif 5'-GAAA-3' (motif A), which contains the sequence motif 5'-CUGANGA-3' (motif B), a reporter molecule and a quencher molecule being attached to each probe molecule, as well as optionally further apparatus and auxiliaries necessary for carrying out the reaction.

29. Kit according to claim 16 , **characterized in that** the reporter is a dye from the group consisting of FAM, HEX, TET, ALEXA, Texas Red, Light Cycler Red, IRD 700, CY-7, IRD 41 or La Jolla Blue and the quencher a dye from the group consisting of TAMRA, CY-5, DABCYL, and LCR.

PCT WELTORGANISATION FÜR GEISTIGES EIGENTUM
Internationales Büro

INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

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Veröffentlicht
Mit internationalem Recherchenbericht.

(54) Title: DETECTION OF NUCLEIC ACID AMPLIFIED PRODUCTS

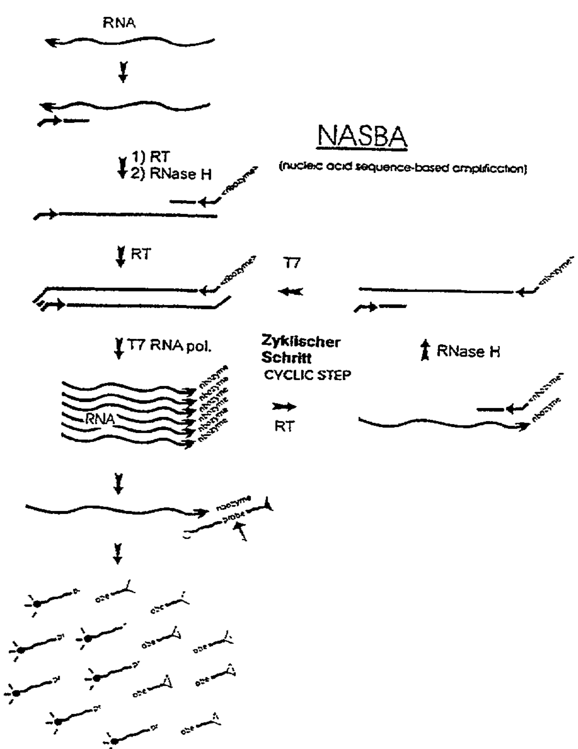
(54) Bezeichnung: DETEKTION VON NUKLEINSÄURE–AMPLIFIKATEN

(57) Abstract

The invention especially relates to methods for the amplification and quantitative real time detection of nucleic acids and to kits for carrying out said method.

(57) Zusammenfassung

Die vorliegende Patentanmeldung betrifft insbesondere Verfahren zur Amplifikation und quantitativen Echtzeitdetektion von Nukleinsäuren sowie Kits zur Durchführung der Verfahren.



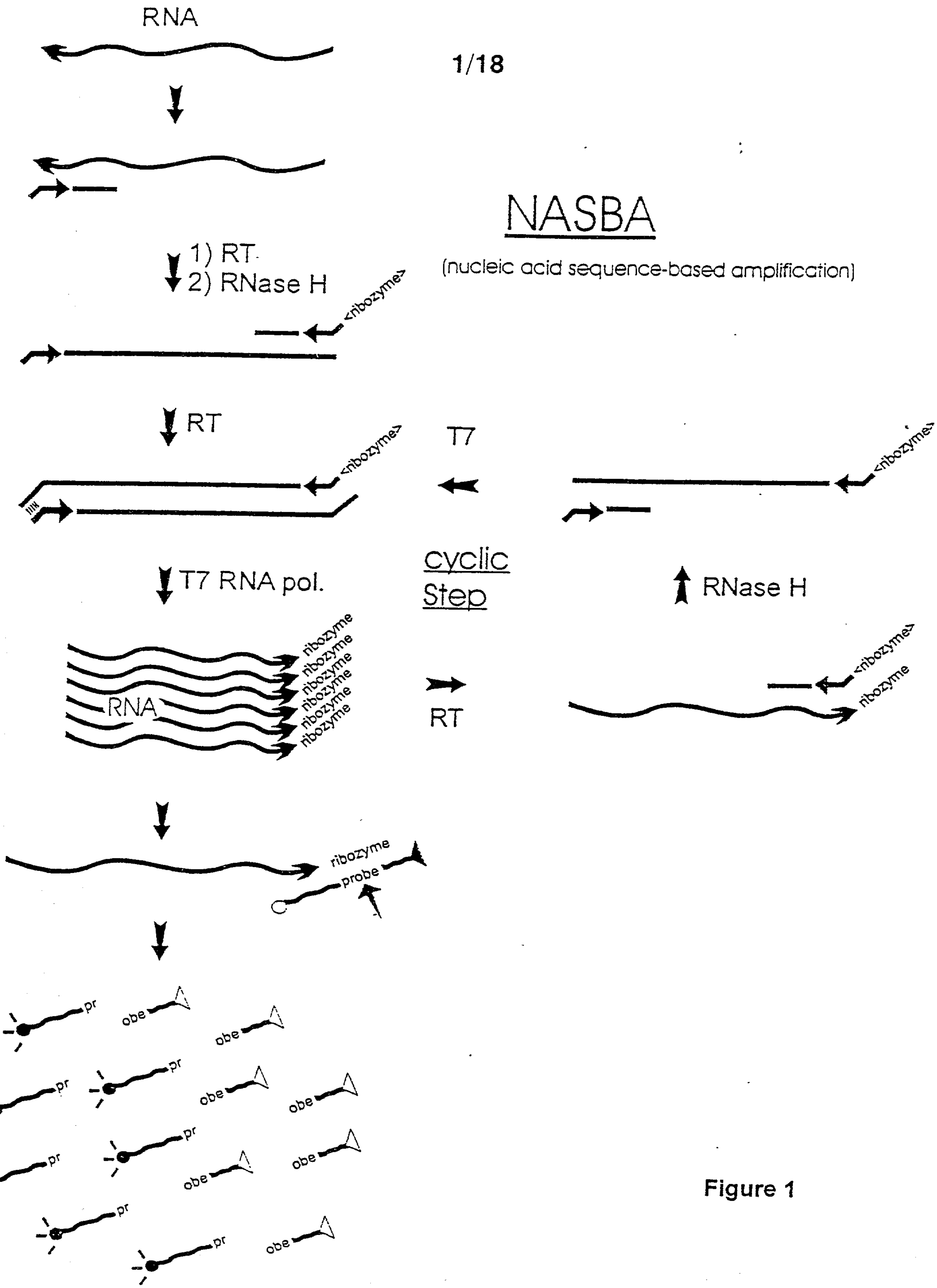

RNA
1) RT
2) RNase H
NASBA
(nucleic acid sequence-based amplification)
<ribozyme>
RT
T7
cyclic Step
T7 RNA pol.
RNase H
ribozyme
RNA
RT
ribozyme
probe
pr
obe

Figure 1

Figure 2A

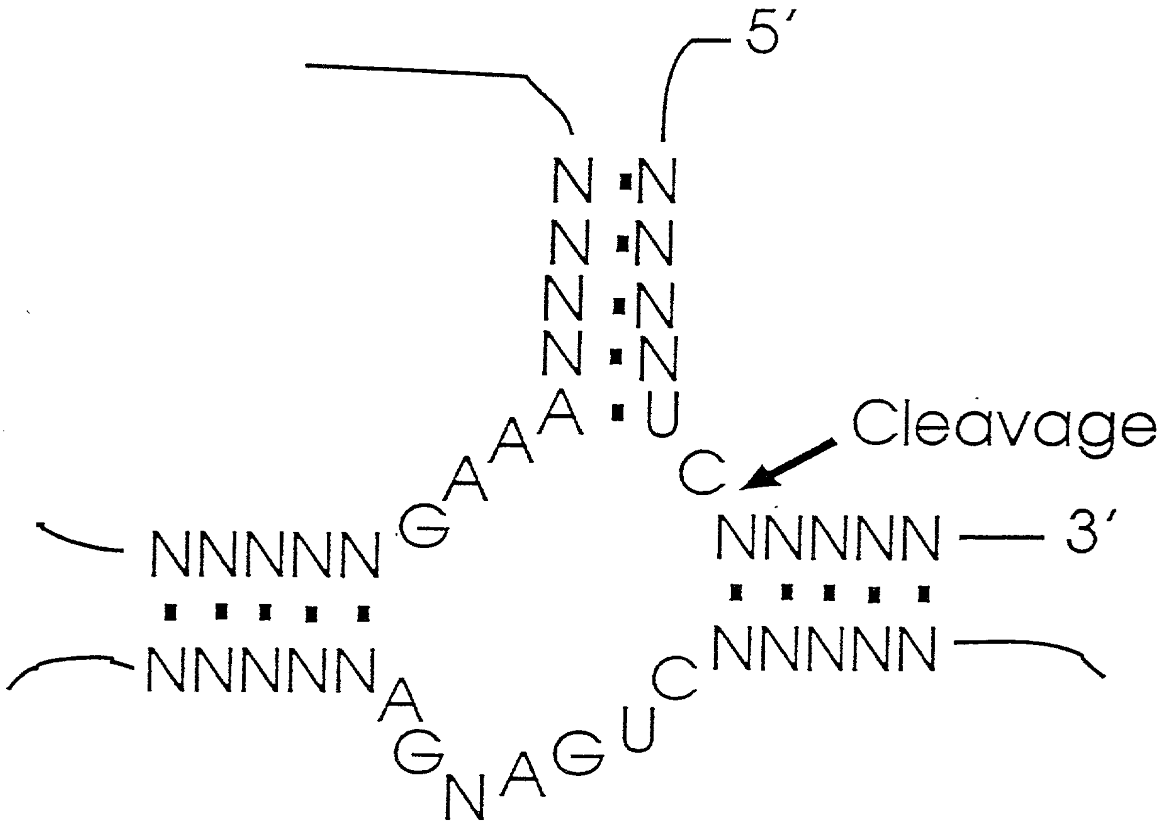
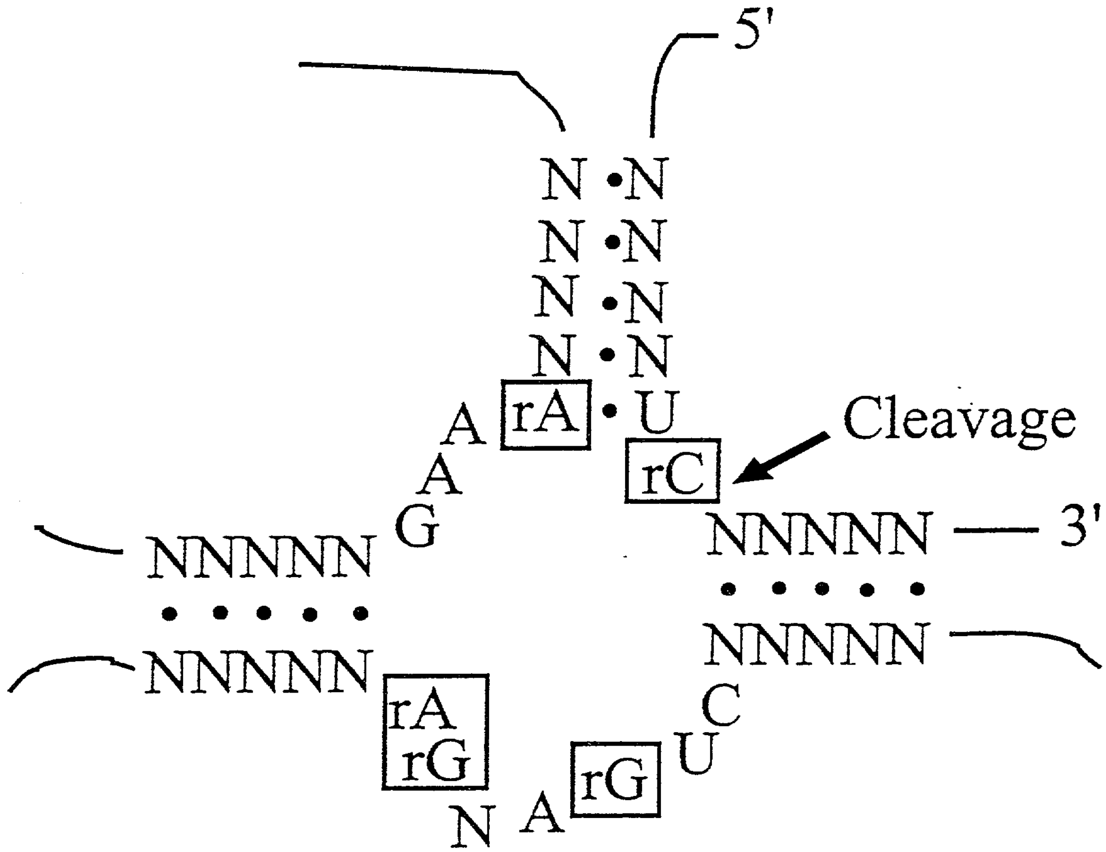

5′
N N
N N
N N
N N
A U
C
Cleavage
NNNNN G A A A
NNNNN — 3′
NNNNN A G N A G U C
NNNNN

Figure 2B


5'
N •N
N •N
N •N
N •N
A rA • U
A rC
G
Cleavage
NNNNN
NNNNN
3'
NNNNN
NNNNN
rA
rG
N
A
rG
U
C

09/937519

Figure 3

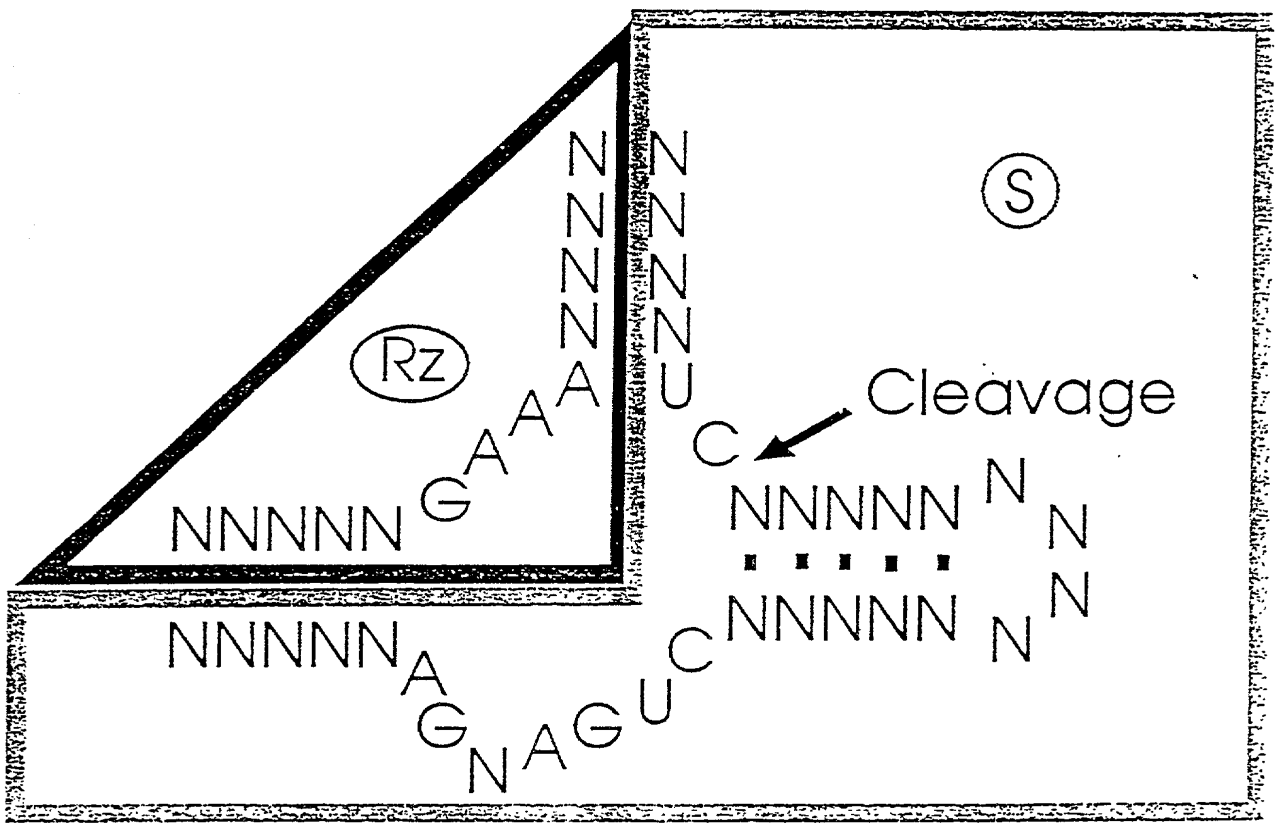
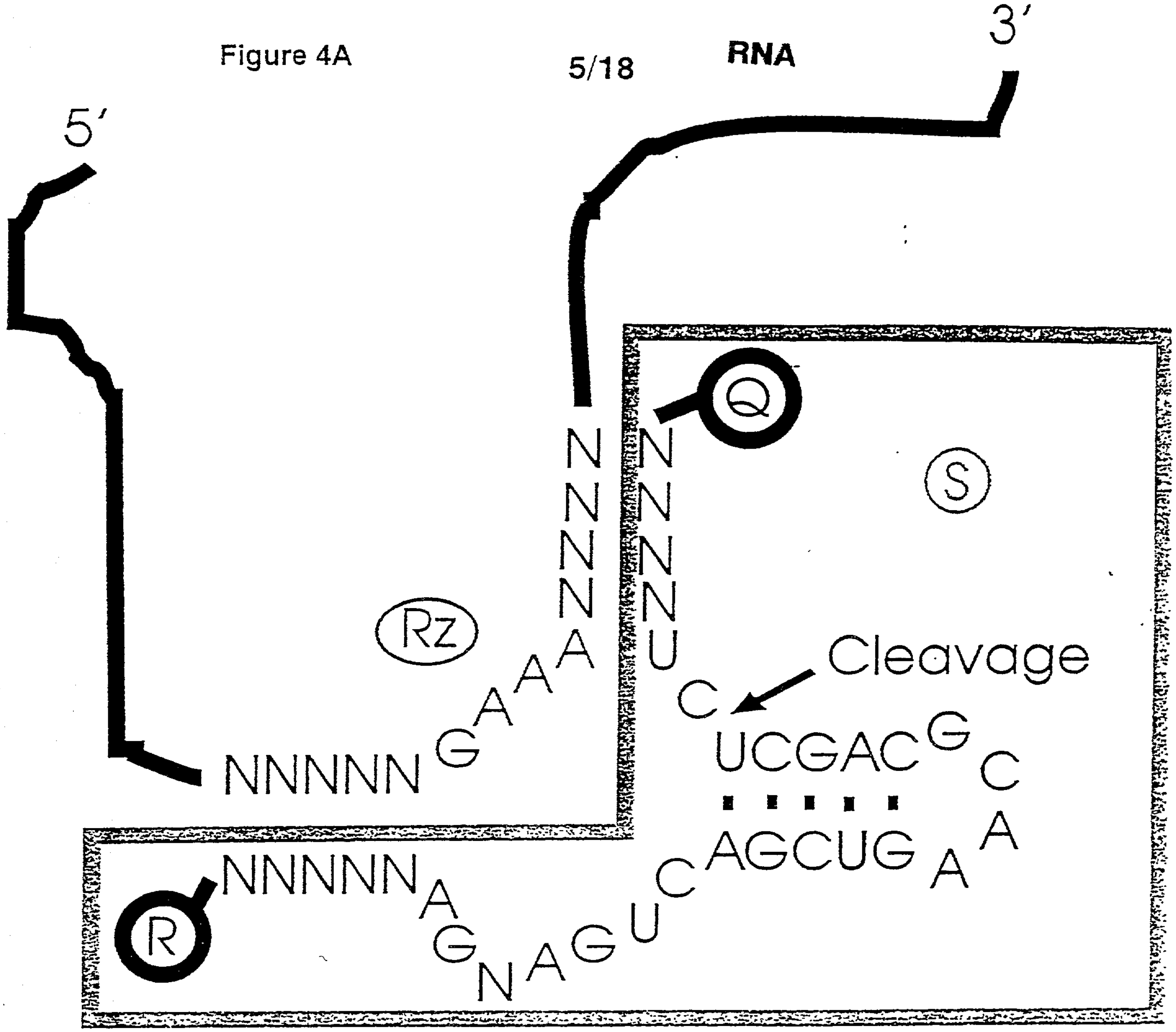


Figure 4A

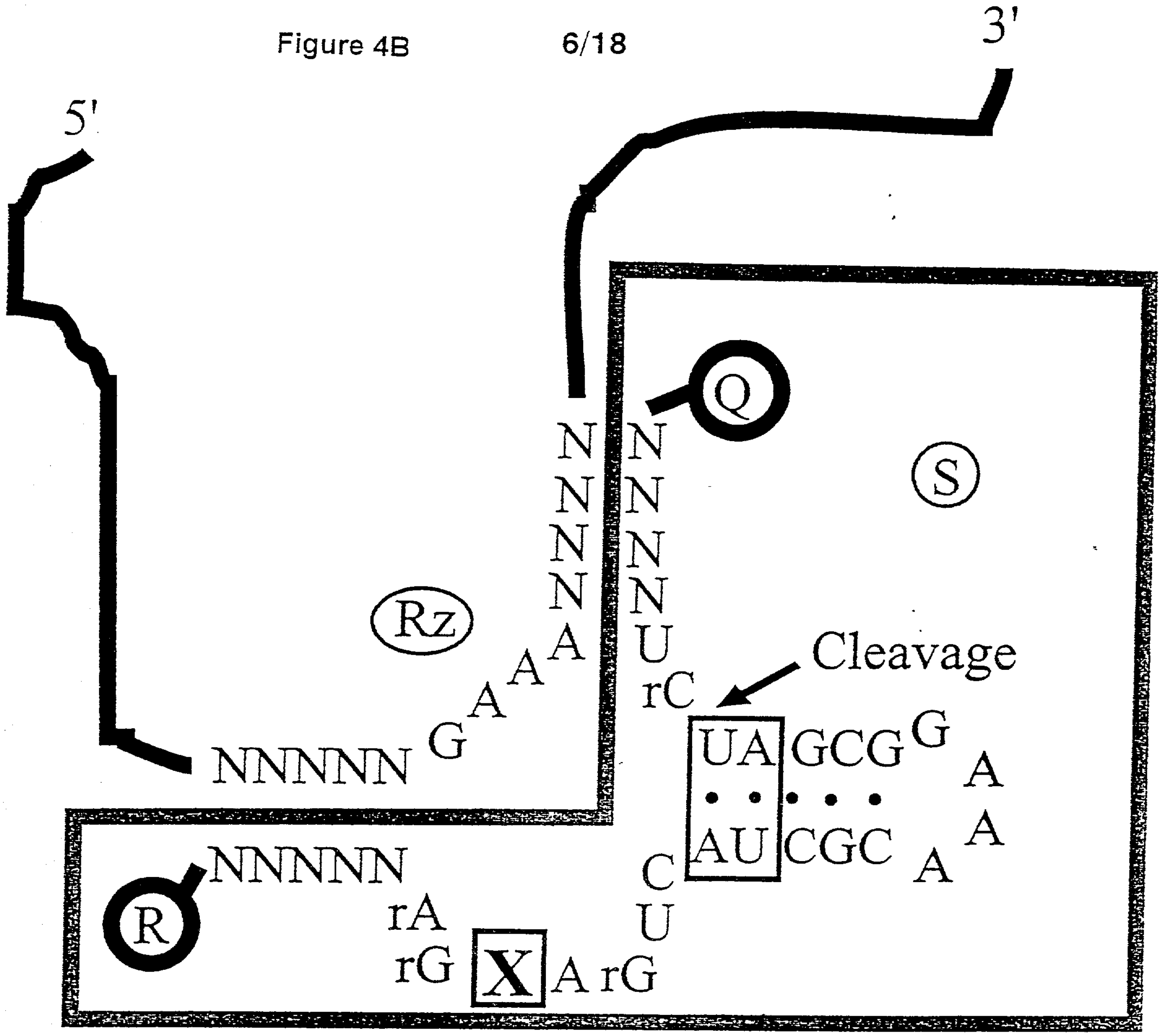


R = FAM
HEX
TET
ALEXA

Q = TAMRA
CY-5
DABCYL
LCR

etc.

Figure 4B



R = FAM
HEX
TET
ALEXA

Q = TAMRA
CY-5
DABCYL
LCR
etc.

X = Pyridin-4-one
r = essential ribonucleotides
important: no (C,U)-A dinucleotide in loop

Figure 5

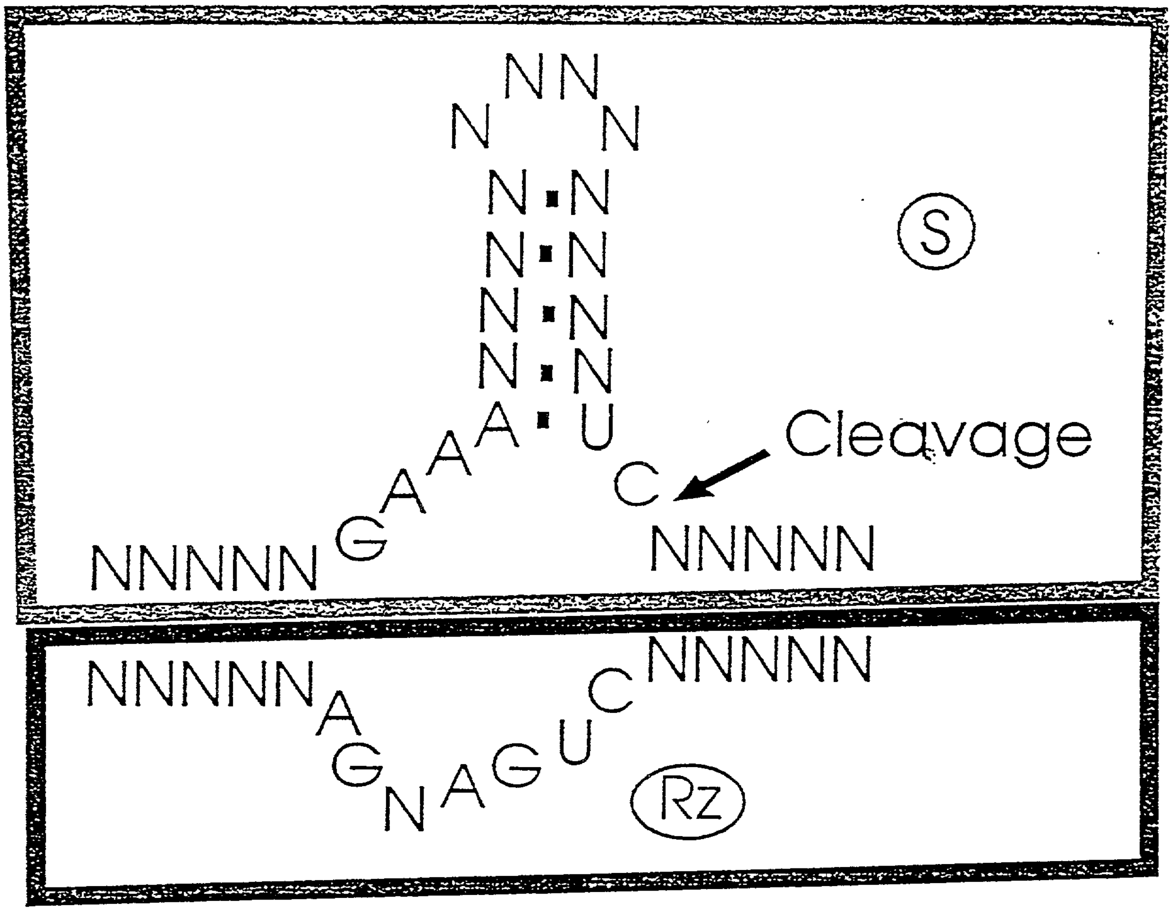
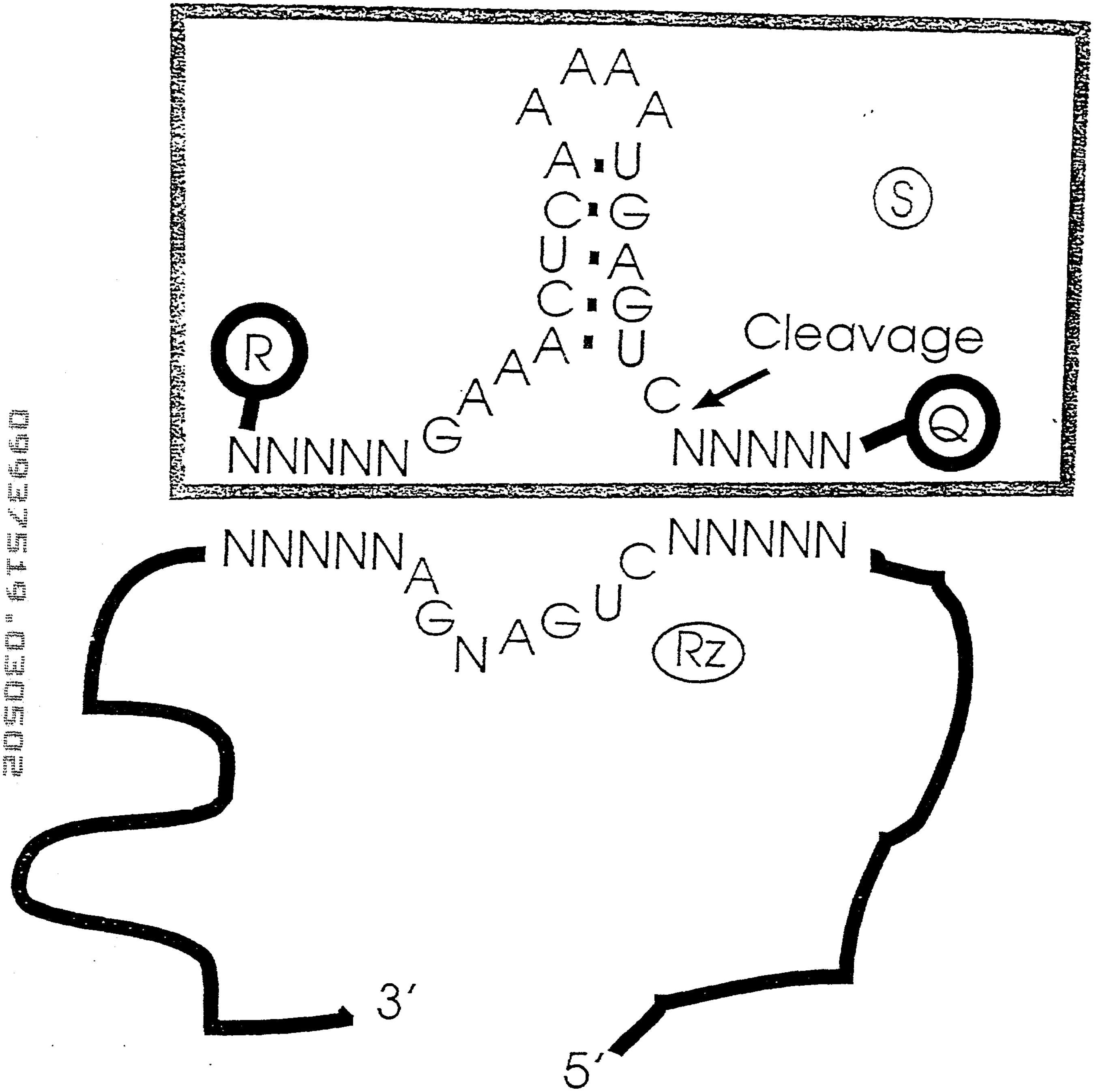

NNNN
N:N
N:N
N:N
N:N
A:U
NNNNNGAAA
C
NNNNN
Cleavage
S
NNNNNAGNAGUC
NNNNN
Rz

Figure 6



AAAA
A U
C G
U A
C G
A U
NNNNN GAAA
C
NNNNN
Cleavage
S
R
Q
NNNNN A G N A G U C NNNNN
Rz
3′
5′

Figure 7

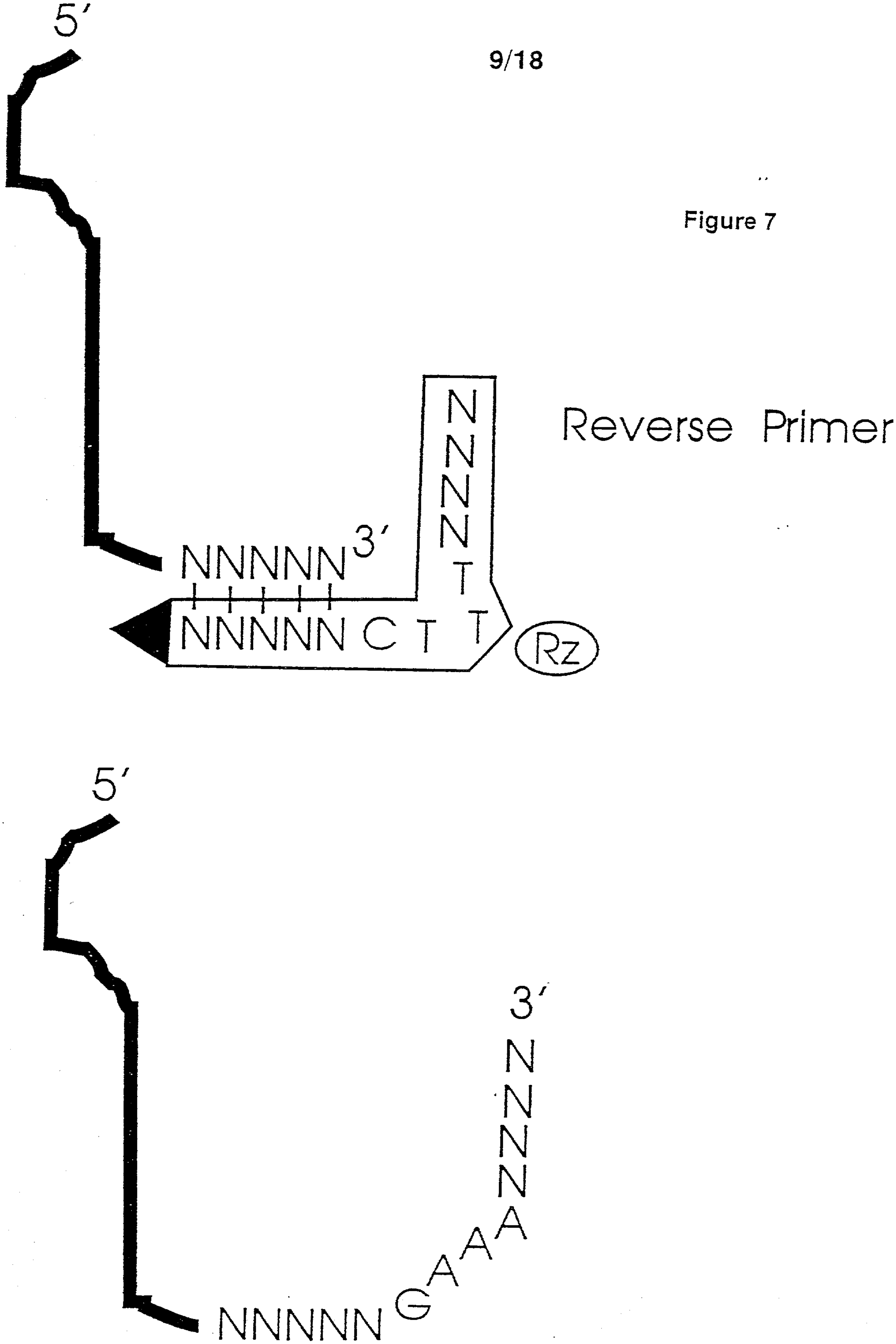
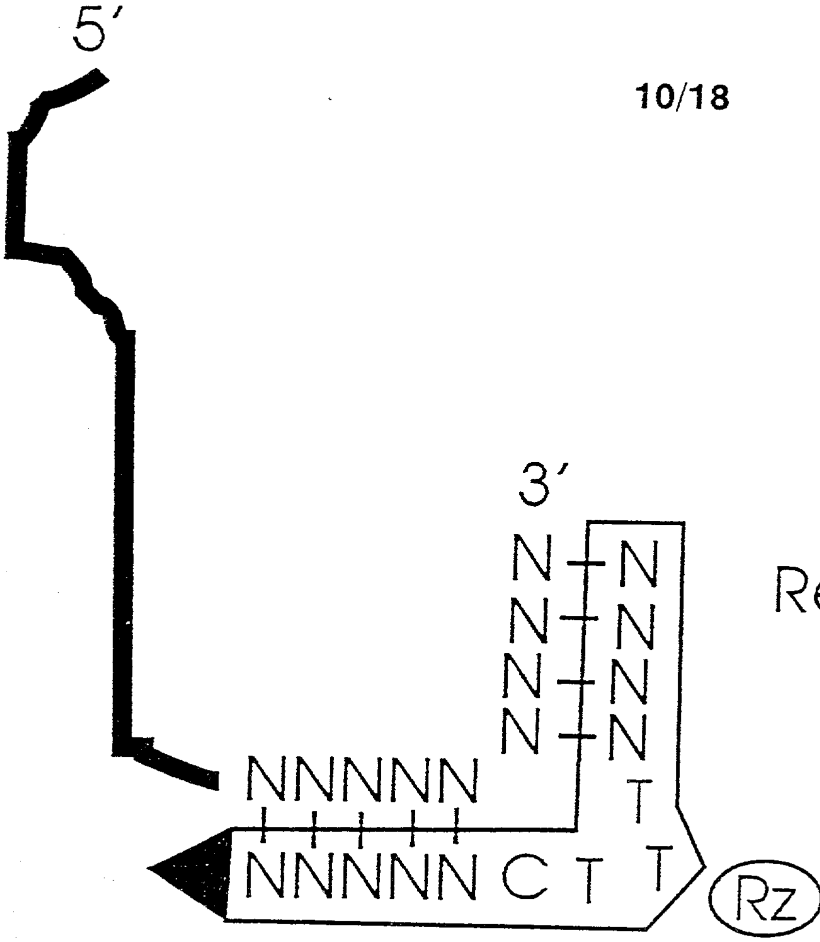

5′
NNNNN 3′
NNNNTT
NNNNN C T T
Rz
Reverse Primer
5′
NNNNN GAAA NNNN 3′

Figure 8



Reverse Primer

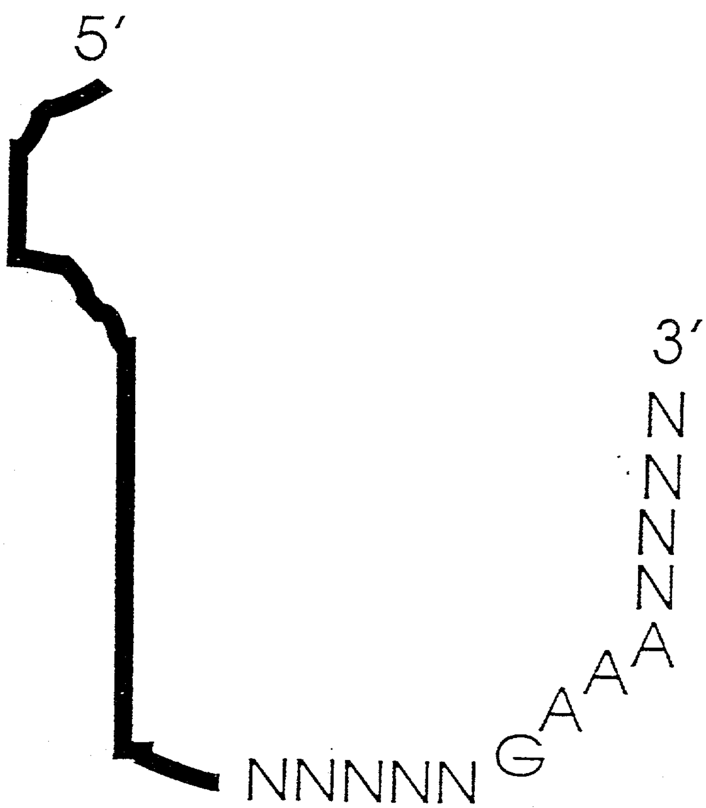
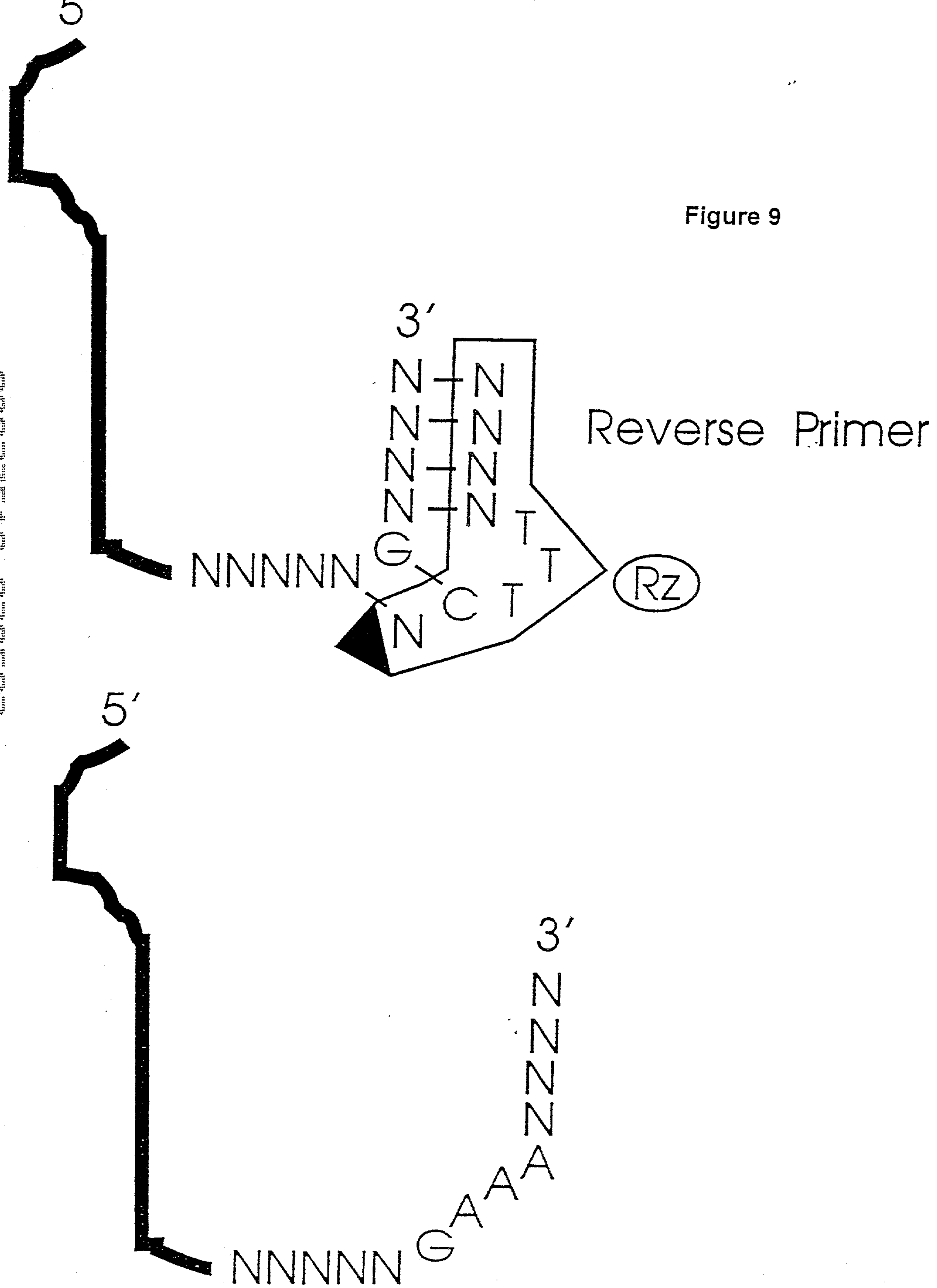


Figure 9


5′
3′
N N
N N
N N
N N
Reverse Primer
NNNNN
G
T
T
C
T
N
Rz
5′
3′
N
N
N
N
A
A
A
G
NNNNN

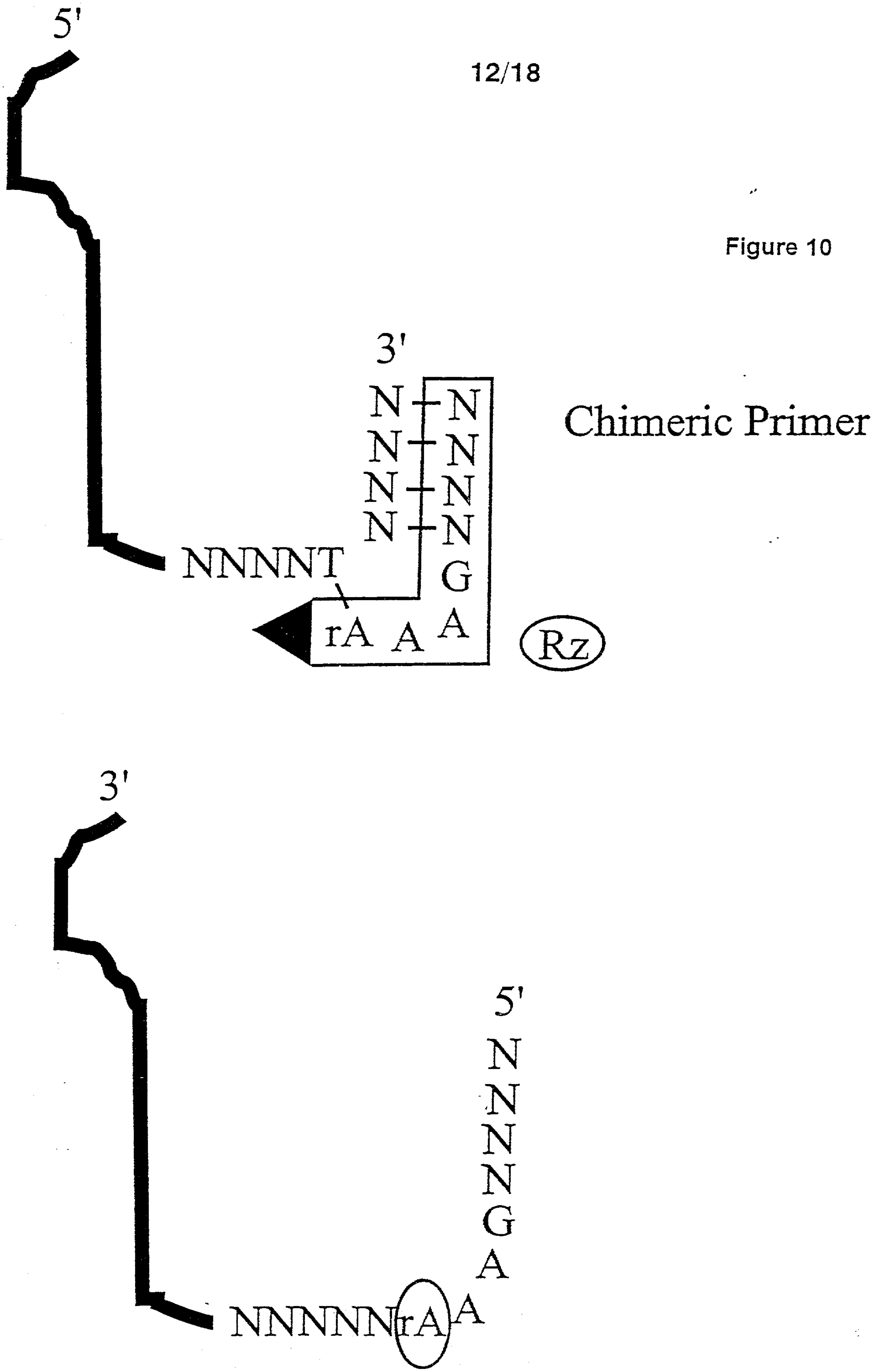

5'
3'
N N
N N
N N
N N
G
NNNNT
rA A A
Rz
Chimeric Primer
3'
5'
N
N
N
N
G
A
A
NNNNNrA

Figure 10

Figure 11

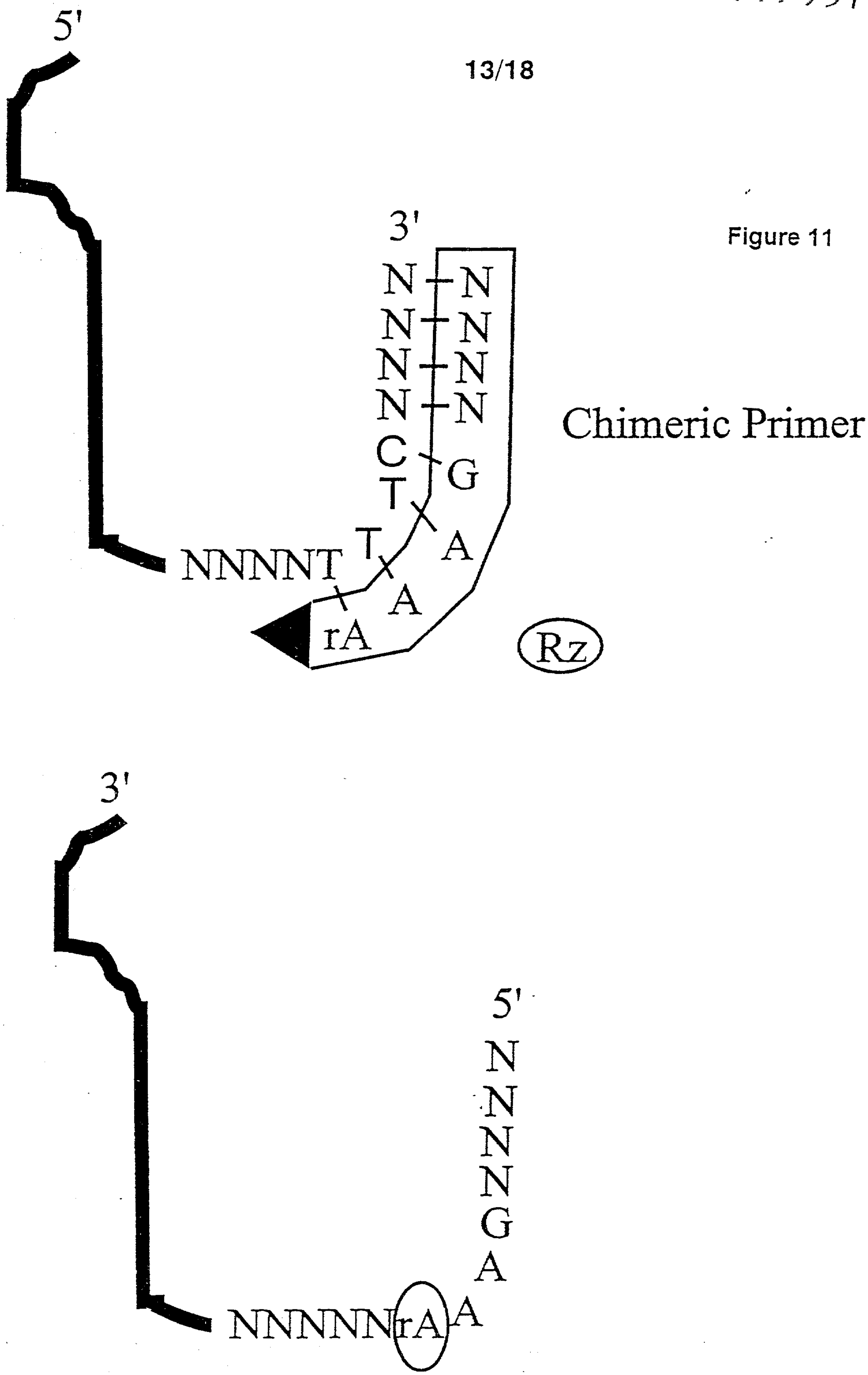
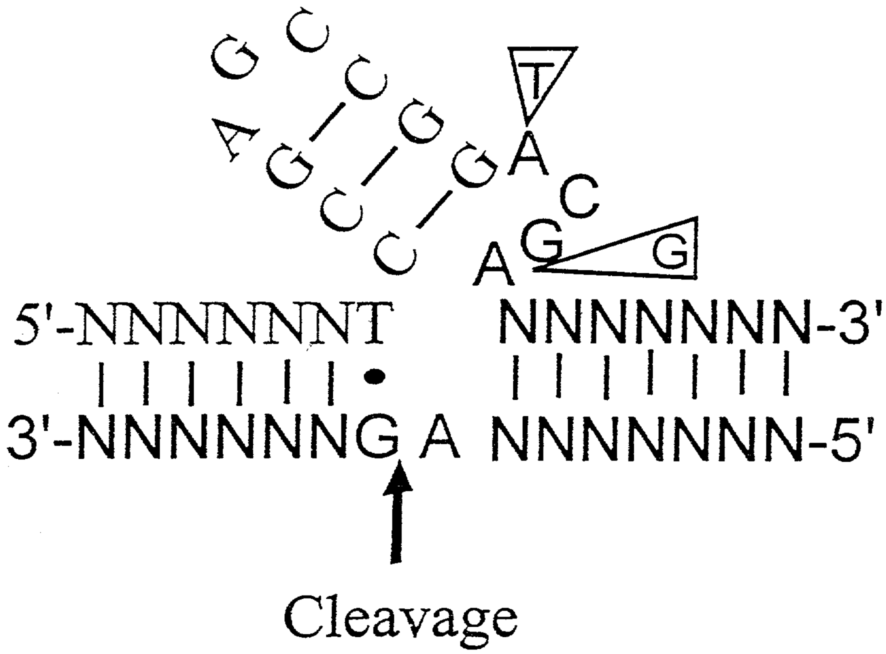
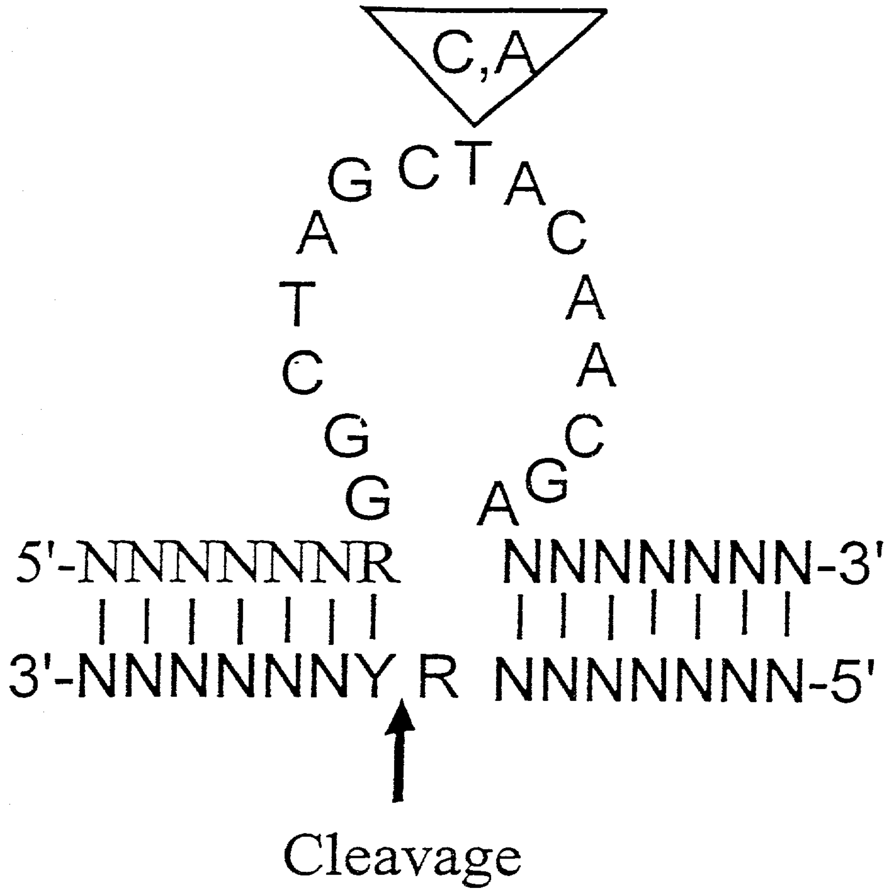

5'
3'
N N
N N
N N
N N
C G
T
T A
A
NNNNT
rA
Chimeric Primer
Rz
3'
5'
N
N
N
N
G
A
A
NNNNNrA

Figure 12



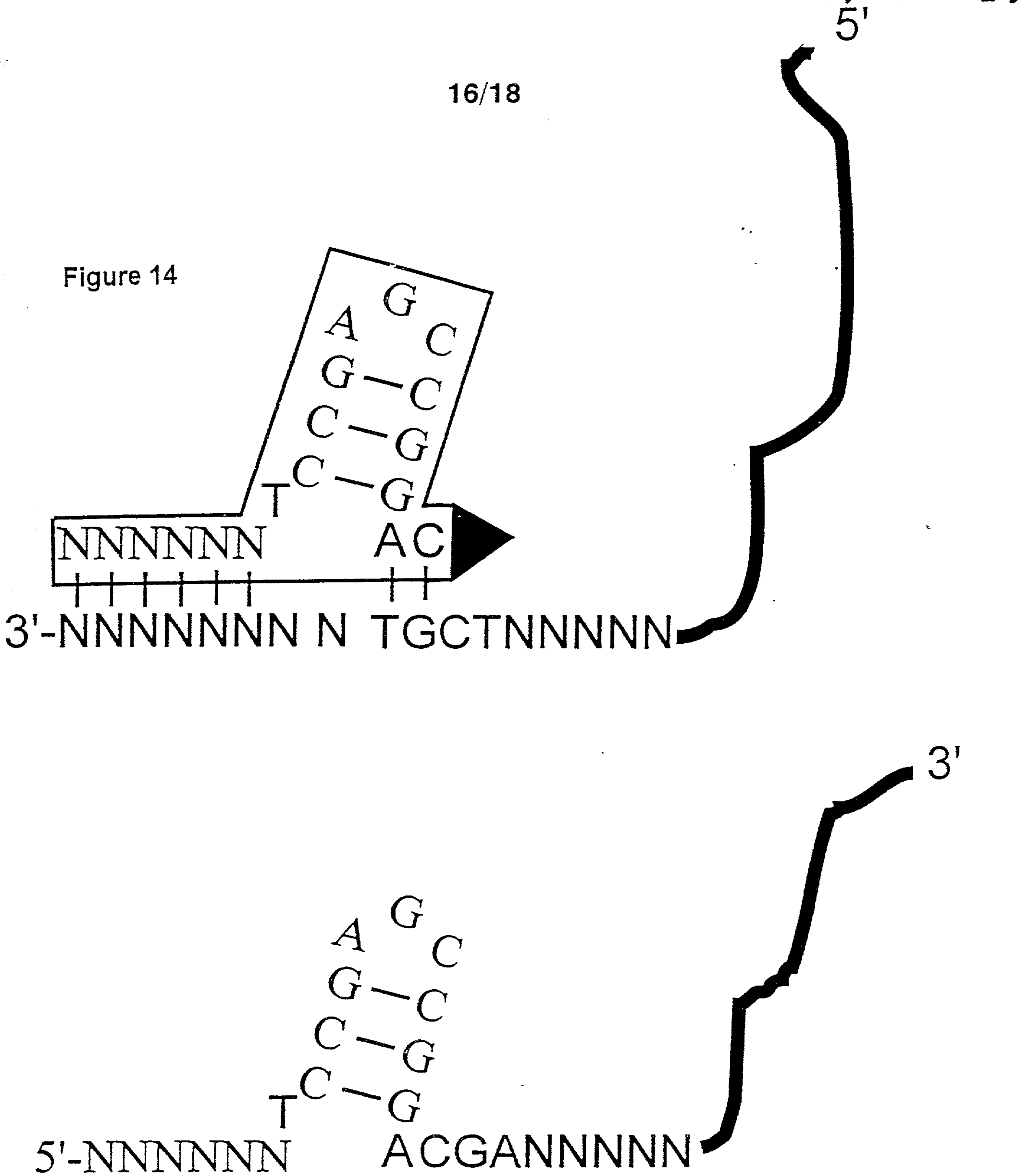
DNA-zyme: Protype A

Figure 13



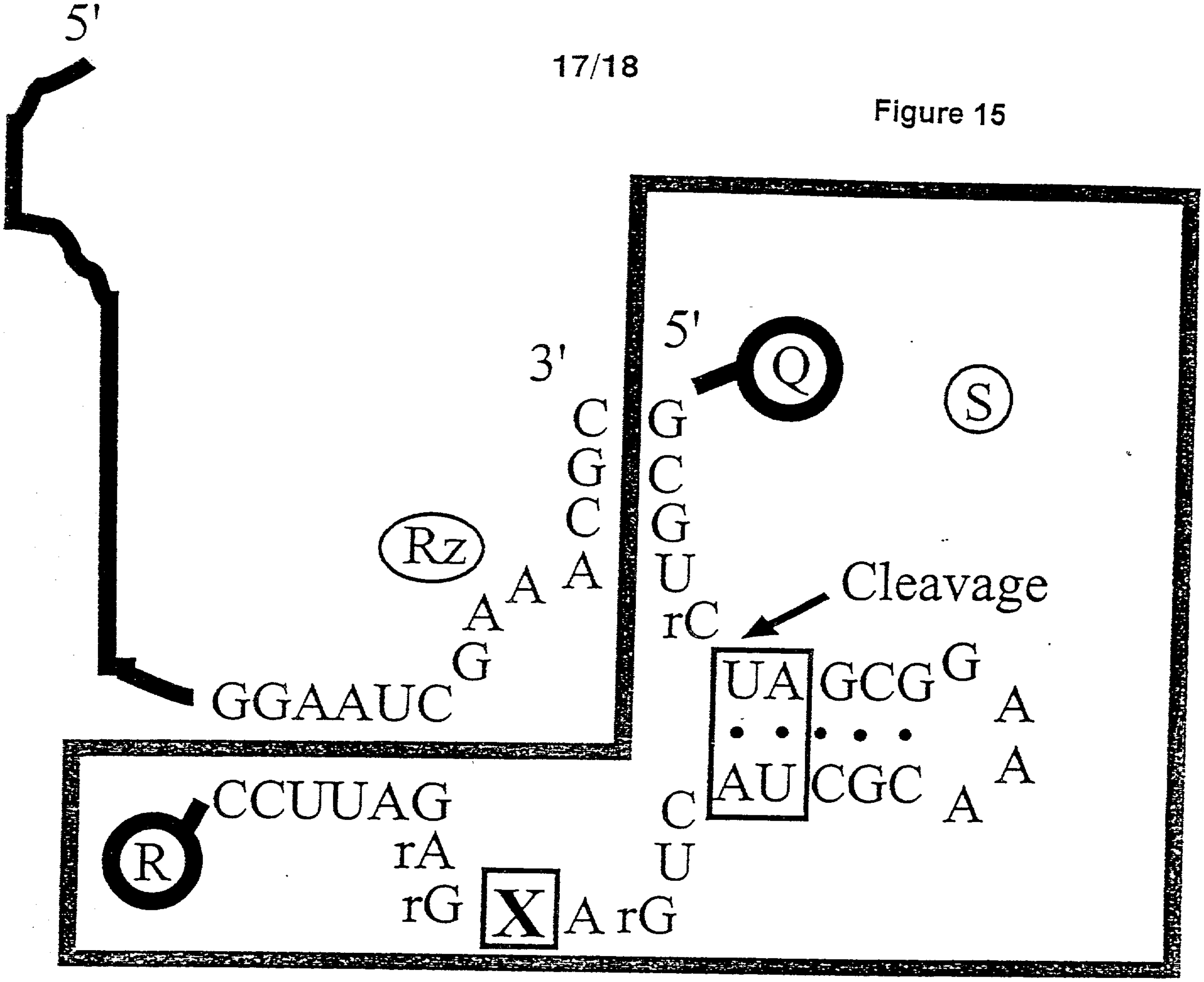
DNA-zyme: Protype B

Figure 14


5'
A G C
G—C
C—G
T C—G
NNNNNN AC
3'-NNNNNNN N TGCTNNNNN
3'
A G C
G—C
C—G
T C—G
5'-NNNNNN ACGANNNNN

Universal Ribozyme Probe

Figure 15



R = FAM
HEX
TET
ALEXA

Q = TAMRA
CY-5
DABCYL
LCR
etc.

X = Pyridin-4-one
r = essential ribonucleotides
important: no (C,U)-A dinucleotide in loop

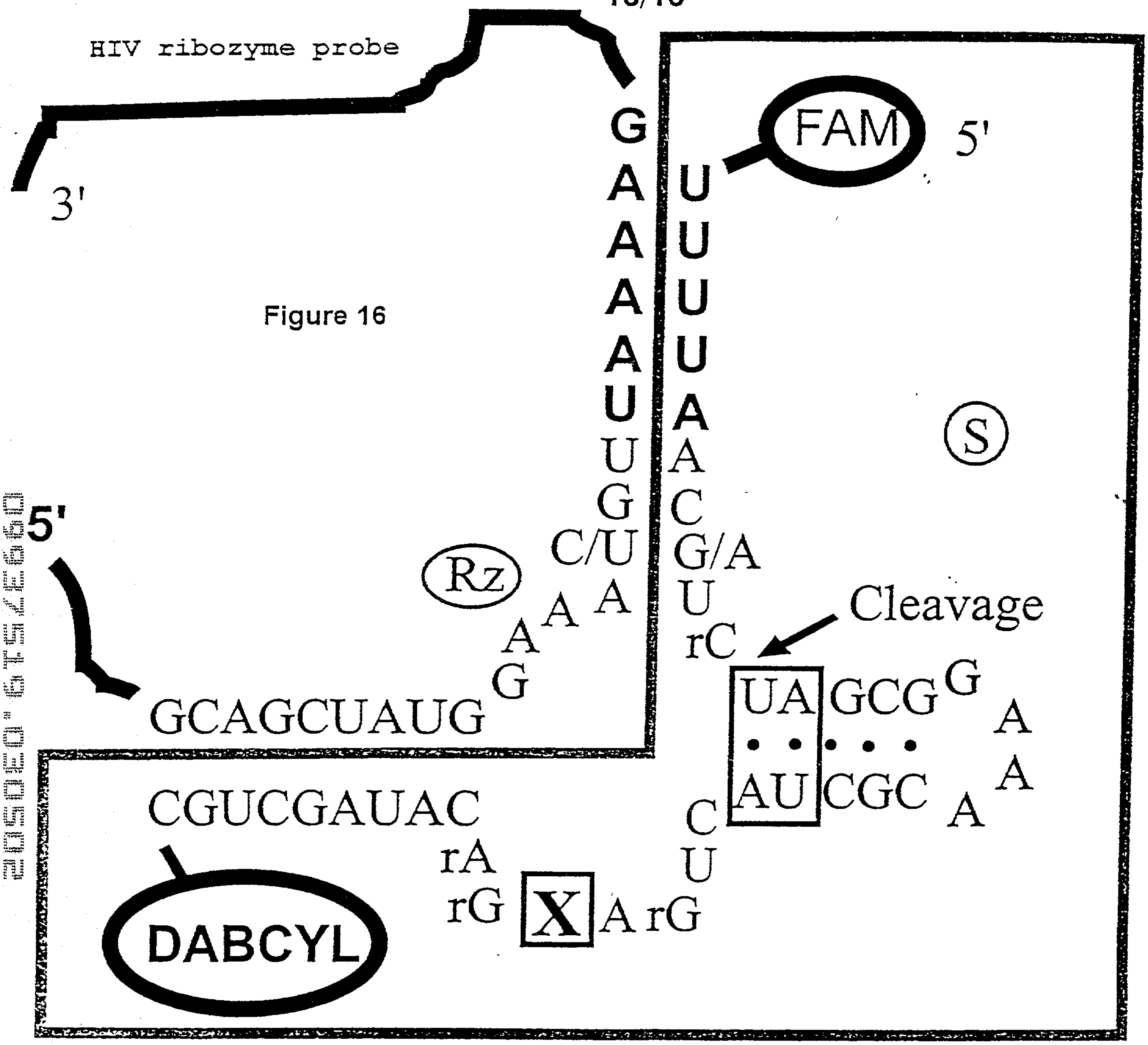


Figure 16

X = Pyridin-4-one

r = essential ribonucleotides

important: no (C,U)-A dinucleotide in loop

DECLARATION

Title: **DETECTION OF NUCLEIC ACID AMPLIFIED PRODUCTS**

International Patent Application No. **PCT/EP99/07127**
International filing date: **September 27, 1999**

Claiming priority to prior **German** application
Serial No. **199 15 141.5**
Filing (priority) date: **March 26, 1999**

Entry into U.S. national stage as Serial No. **09/937,519**
U.S. National Stage entry date: **September 25, 2001**
As amended with English PCT claims filed therewith
U.S. Applicant / Inventor: **Guido Krupp**

Campbell & Flores Attorney Docket No. **P-UX 4977** (**P 58130**)

I believe that I am the original and first inventor of the subject matter that is claimed and for which a patent is sought in the application identified above.

I hereby state that I have reviewed and understand the contents of the application identified above, including the specification and claims.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to myself to be material to patentability as defined in Title 37, Code of Federal Regulations, Sec. 1.56.

Under Sec. 1.56, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and (1) It establishes, by itself or in combination with other information, a prima facie

case of unpatentability of a claim; or (2) It refutes, or is inconsistent with, a position the applicant takes in: (a) Opposing an argument of unpatentability relied on by the U.S. Patent and Trademark Office, or (b) Asserting an argument of patentability.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

My citizenship, residence and mailing address are correctly stated below my name:

1-00

Full name of inventor: **Guido Krupp**

Citizenship: **Germany**

Residence: **Kiel, Germany** DET

Rosenstr. 3, D-24622 Gnutz

Mailing Address: ~~**Wannseebogen 30A, D-24111 Kiel**~~**, Germany**

11.12.2001

G. Krupp

Signature: G. Krupp Date: 11.12.2001

POWER OF ATTORNEY

Assignee **Artus Gesellschaft für molekularbiologische Diagnostik und Entwicklung mbH**

is the owner of the entire right, title and interest of the following U.S. patent application identified below and any subsequently filed divisional, continuation, continuation-in-part or reissue applications claiming priority thereto.

Title: **DETECTION OF NUCLEIC ACID AMPLIFIED PRODUCTS**

International Patent Application No. **PCT/EP99/07127**
International filing date: **September 27, 1999**

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U.S. National Stage entry date: **September 25, 2001**
As amended with English PCT claims filed therewith
U.S. Applicant / Inventor: **Guido Krupp**

Campbell & Flores Attorney Docket No. **P-UX 4977 (P 58130)**

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